REVIEW

Astonishing Diversity of Natural Surfactants: 7. Biologically Active Hemi- and Monoterpenoid Glycosides

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ABSTRACT: This review article presents 90 hemi- and 188 monoterpenoid glycosides, isolated and identified from plants and microorganisms, that demonstrate different biological activities. These natural bioactive glycosides are good prospects for future chemical preparations from these compounds as antioxidants and as anticancer, antimicrobial, and antibacterial agents. These glycosidic compounds have been subdivided into several groups, including hemiterpenoids; acyclic, monocyclic, and bicyclic monoterpenoids; and iridoid monoterpenoids.

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Terpenes (terpenoids) are the second-largest group of secondary metabolites (nearly 23,000 are known). They are incredibly diverse in structure and activity, even though they all originally derive from a simple molecule called isoprene. Many terpenes are hydrocarbons, but oxygen-containing compounds such as alcohols, aldehydes, or ketones (terpenoids) are also isolated from natural sources. Their building block is the hydrocarbon isoprene CH_2=C(CH_3)–CH=CH_2. The German chemist Otto Wallach was the first to propose that monoterpenoids were constructed of a linkage of isoprene units (in head-to-tail form) (2). Wallach (born March 27, 1847, in Königsberg, died February 26, 1931, in Göttingen) was awarded a Nobel Prize in Chemistry in 1910. His interest began with the analysis of fragrant essential oils—oils removed from plants by steam distillation, with industrial uses; he then started researching their molecular structure. Wallach succeeded in determining the structure of several terpenes, including limonene, in 1895 (3,4). He showed that terpenes were derived from isoprene, C_5H_8—his "isoprene rule"—and therefore had the general formula $(C_5H_8)_n$.

Such compounds were classified by molecular size in a way that was systematic, but not consistent with the idea of isoprene as the structural unit. Later, Leopold (Lavoslav) Ruzicka (pronounced closely following the French transcription, "Rougitchka"; born on September 3, 1887, in Vukovar, Croatia; died on September 26, 1976, in Zürich, Switzerland) (5,6) formulated a classification system in which he defined monoterpenes as having carbon skeletons with two 5-carbon isoprene units, sesquiterpenes with three isoprenes, and so on. In 1953 his "biogenetic isoprene rule," which was pioneered by Wallach, became the crowning achievement of his lifetime. Ruzicka's main work, started in 1921, involved macrocyclic compounds, higher terpenes, and steroids. He shared the Nobel Prize with Adolf Friedrich Johann Butenandt (1903–1995) in 1939.

The hemiterpene isoprene, which contains five carbons (one isoprene unit, C_5), is a gas emitted into the atmosphere by many plant species. A monoterpene (monoterpenoid) contains 10 carbons (two isoprene units, C_{10}); a sesquiterpene, 15 carbons (three isoprene units, C_{15}); and a diterpene, 20 carbons (four isoprene units, C_{20}). Sesterpenes (five isoprene units, C_{25}) were isolated from insect protective waxes and from fungal sources. Triterpenes (six isoprene units, C_{30}) are important structural components of plant cell membranes. Many plant pigments, including the yellow and red carotenoids, are tetraterpenes (eight isoprene units, C_{40}). Natural rubber is a polyterpene containing more than 40 isoprene units (7,8).

Terpenes are largely found in essential oils, and they were known and used in ancient Egypt for various religious aims. The terpene camphor, obtained from the camphor tree (*Cinnamomum camphora* syn. *Laurus camphora*, Lauraceae) was used to reduce fevers, soothe gums, and treat epilepsy. Camphor trees are native to China and Japan and are cultivated for their wood for the extraction of camphor oil. Marco Polo was the first to note that the Chinese used camphor oil as a medicine, scent, and embalming fluid. Camphor crystals have strong antiseptic, stimulant, and antispasmodic properties and are applied externally as unguents or balms as a counterirritant and analgesic liniment to relieve arthritic and rheumatic pains, neuralgia, and back pain. It may also be applied for skin problems such as cold sores and chilblains, and used as a chest rub for bronchitis and other chest infections. Camphor was introduced in Europe from the East by the Arabs around the 11th century. The process of obtaining plant essential oils by fat extraction was known by the early Middle Ages. These compounds have important uses as flavorings and perfumes, as well as intermediates in the production of other commercial products such as solvents and adhesives. Many terpenes play roles as plant hormones and in the chemical defenses of plants against microbial diseases and insect herbivores; many others have important medicinal properties. Many terpenoids and isoprenoids are toxic to insects, and a few are also repellents and toxicants to subterranean termites, although the mechanism of toxicity is not well known.

Iridoids, a widely distributed class of natural monoterpenoids, have shown encouraging biological activities including hepatoprotective, anticancer, immunostimulant, and antileishmanial activities (9,10).

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For the previous article in this series, see Reference 1.

Abbreviations: IPP, isopentenyl diphosphate; MEP, methylerythritol phosphate.

Hemi- and monoterpenoid glycosides are representatives of a water-soluble group isolated from terrestrial and marine plants and organisms. They can be subdivided into several known groups including hemiterpenoids, monoterpenoids (acyclic, monocyclic, and bicyclic), and iridoid monoterpenoids and are presented in this review article.

HEMITERPENOID GLYCOSIDES AND RELATED COMPOUNDS

A small group of terpenes named hemiterpenes are made up of one 5-carbon unit (or from C5 to C9), and are the simplest of all terpenes. Isoprene is emitted from the leaves of many plants, and experimental data have been partly reviewed in some articles (11–15). Hemiterpenes accumulate in plant tissues and can be found in association with other compounds such as alkaloids, coumarins, phenols, and/or flavonoids (16,17).

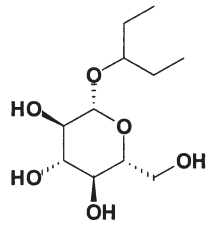
Plants have been shown to use the mevalonate pathway for the biosynthesis of sterols and triterpenes in the cytoplasm and to use the recently discovered deoxyxylulose phosphate pathway for the biosynthesis of a variety of hemiterpenes, monoterpenes, and diterpenes, as well as for the biosynthesis of carotenoids and the phytol side chain of chlorophyll in plastids (18). In higher plants, the five-carbon building blocks of all terpenoids, isopentenyl diphosphate (IPP), and dimethylallyl diphosphate are derived from two independent pathways localized in different cellular compartments (19). The methylerythritol phosphate (MEP, or nonmevalonate) pathway, localized in the plastids, is thought to provide IPP and dimethylallyl diphosphate for hemiterpene, monoterpene, and diterpene biosynthesis, whereas the cytosol-localized mevalonate pathway provides C_5 units for sesquiterpene biosynthesis. It has been shown that only one of the two pathways, the plastid-localized MEP pathway, is active in the formation of volatile terpenes. The MEP pathway provides IPP precursors for both plastidial monoterpene and cytosolic sesquiterpene biosynthesis in the epidermis of snapdragon petals. The trafficking of IPP occurs unidirectionally from the plastids to the cytosol. The MEP pathway operates in a rhythmic manner controlled by the circadian clock, which determines the rhythmicity of terpenoid emission (19). Different Salicaceae species contain from 2 to 8% hemiterpenes of the total volatile oil (20).

Foeniculum vulgare, the common fennel, is a biennial or perennial plant with a whitish, tap-shaped root, the whole herb being smooth and of a deep glaucous green. It inhabits the southern parts of Europe and is naturalized in Japan, Kurdistan, Malaysia, Mexico, Spain, Turkey, and Venezuela. For the medicinal use of its fruit (commonly called seeds), fennel is largely cultivated in the south of France, Saxony, Galicia, and Russia, as well as in India and Persia. Fennel was well known to the ancients and was cultivated by the ancient Romans for its aromatic fruit and succulent, edible shoots.

The *F. vulgare* fruit extract exhibited anti-inflammatory, analgesic, and antioxidant activities (21) and showed acaricidal activities against *Dermatophagoides farinae* and *D. pteronyssinus* (22) as well as antimicrobial activity against some phytopathogenic bacterial species (*Pseudomonas syringae, P. cichorii, P. viridiflava, P. corrugate, P. tolaasii, P. reactans, P. agarici, Erwinia carotovora* subsp. *carotovora, Agrobacterium tumefaciens, Burkholderia gladioli* pv. *agaricicola,* and *Xanthomonas campestris*) (23). Some hemiterpenoid glycosides (**1–9)** were obtained from the water-soluble fraction of the methanol extract of the fennel fruit (*F. vulgare*, Umbelliferae) (24).

A new hemiterpene glucoside, (2*E*)-4-hydroxy-2-methyl-2-butenyl β-D-glucopyranoside **10**, was isolated from Italian populations of *Ornithogalum montanum* (Liliaceae) (25). Four new nonbasic hemiterpenoid glucosides, woorenosides VI **12**, VII **13**, VIII **11**, and IX **14**; the nonglycosidic woorenosides X and XI; and a new acetylated flavone glycoside, woorenoside XII, were isolated from the fresh rhizomes of *Coptis japonica* var. *dissecta* (26). (*S*)-2-Methylbutan-1-yl-β-D-glucopyranoside **15** was isolated in a yield of 0.01% from the leaves of *Bystropogon plumosus* (Lamiaceae) endemic to the Canaries (27). The essential oil of some species of the genus *Bystropogon—B. plumosus*, *B. origanifolius* var. *palmensis*, *B. wildpretii*, *B. maderensis*, and *B. canariensis* var. *smithianus*—showed antimicrobial and antifungal activities (28). A new hemiterpenoic acid glycoside named securiterpenoside **16** was isolated from the Chinese medicinal plant *Securidaca inappendiculata* (Polygalaceae), whose bark is used for bathing and shampooing the hair (29).

Two new hemiterpene glycosides were isolated from wine of the grape *Vitis vinifera* cv. Gewurztraminer and were identified by MS and NMR spectroscopy as *O*-β-D-apiofuranosyl-(1→6)-*O*-β-D-glucopyranosides of 3-methyl-3-butenol **17** and of 3-methyl-2-butenol **18** (30). Two hemiterpene glycosides, (2*Z*)-3-hydroxy-1-methyl-1-propenyl-β-D-glucopyranoside **19** and (2*Z*)-3-hydroxy-2-methyl-1-propenyl-β-D-glucopyranoside **20**, were identified in a water-soluble extract obtained from Riesling grapevine leaves and purified by HPLC (31).



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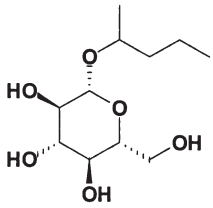

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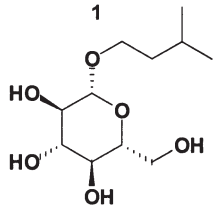

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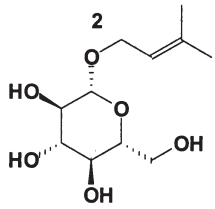

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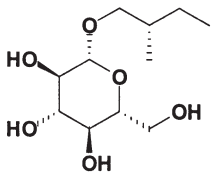

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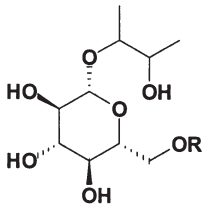

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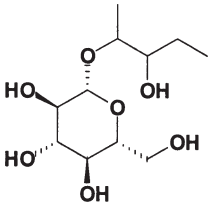
7 R = Api



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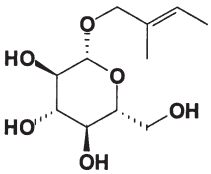

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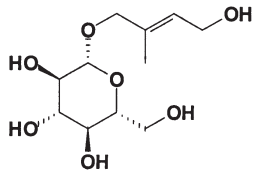

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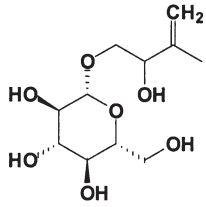

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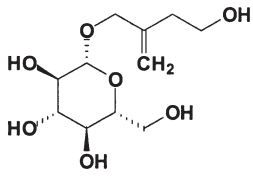

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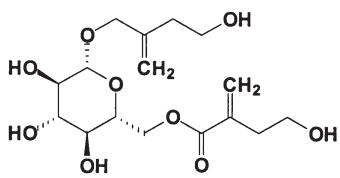

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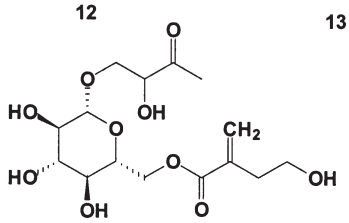

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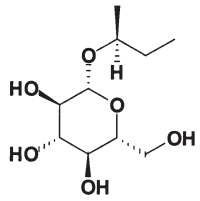

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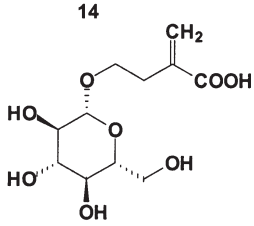

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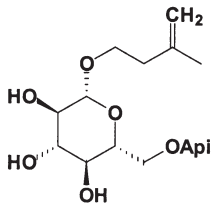

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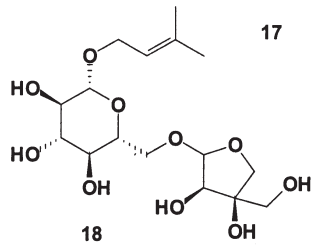

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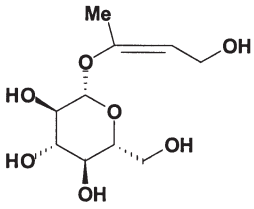
Ilex (holly) is a genus of about 400 species of flowering plants in the family Aquifoliaceae. *Ilex* is the old Latin name for holly (*Ilex aquifolium*) or the aohada holly (*Ilex macropoda*) now being used in medicine (32). Extracts from species of the genus *Ilex* showed proteasome inhibitor activity (33) and were capable of inhibiting advanced glycation end products (34). Phytochemical studies of *I. macropoda* led to the isolation of four new hemiterpene glycosides—(2*E*)-4-hydroxy-2-(hydroxymethyl)-2-butenyl β-D-glucopyranoside **21**, aohadaglycoside A **22**, aohadaglycoside B **23**, and aohadaglycoside C **24**—identified from a bark extract (35).



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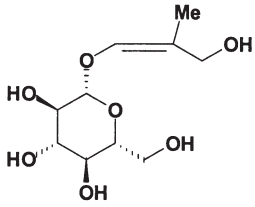

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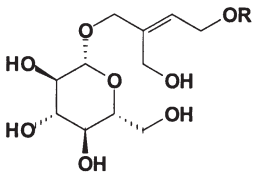

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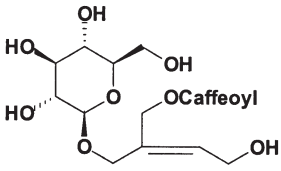

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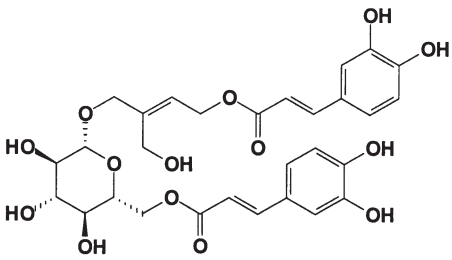

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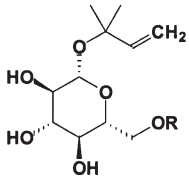

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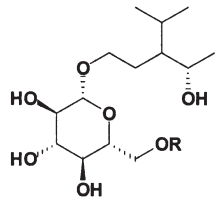

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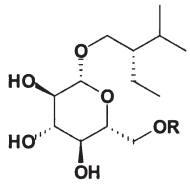

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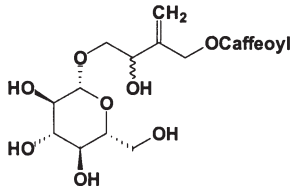

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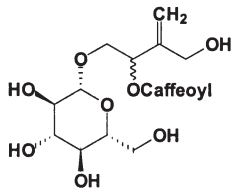

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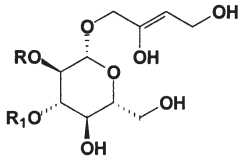
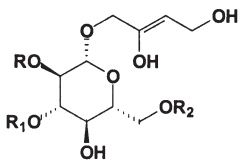
The hemiterpenoid glucoside 1,1-dimethylallyl β-glucoside **25** was isolated from flower buds of *Musa paradisiacal* (36), and furocoumarin **26**, having a similar structure, was isolated from a methanolic extract of the root and rhizoma of *Glehnia littoralis* (Umbelliferae; Hamabofu in Japanese) (37). A methanolic extract of the roots of *Streptocaulon juventas* showed strong antiproliferative activity against the highly metastatic human HT-1080 fibrosarcoma cell line; two new hemiterpenoids, (4*R*)-4-hydroxy-3-isopropylpentyl β-rutinoside **27** and (*R*)-2-ethyl-3-methylbutyl β-rutinoside **28**, were isolated from this extract (38). Two new hemiterpene glucosides named pubescenosides A **29** and B **30** were isolated from the root of *Ilex pubescens* (39). A pharmacological investigation of pubescenosides A and B indicated that both possessed potent antiplatelet aggregation activities.

Acylated hemiterpenoid glycosides called hymenosides A **31**, B **32**, C **33**, D **34**, E **35**, and F **36**, where HPA = 4-hydroxyphenylacetate, were isolated from a methanolic extract of the Japanese fern *Hymenophyllum barbatum* belonging to the family Hymenophyllaceae (40). Hymenosides K **37**, Q **38**, R **39**, S **40**, L **48**, M **43**, N **45**, O **46**, P **47**, W **44**, and U **49** and the methyl ester of 3-(β-D-glucopyranosyloxy)-5-hydroxy-hexanoic acid, **50**, were identified from the same species (41). The structures of those aglycons were divided into four types: 2-methyl-but-2-ene-1,4-diol, 2-hydroxymethyl-but-2-ene-1,4-diol, 2-methylene-butane-1,3,4-triol, and 3-hydroxy-5-hexanolide. The sugar moieties, which were acylated by phenylacetic acid derivatives, were also established by chemical and spectroscopic methods. Eight glucosides of the isolated compounds had a bitter or weakly pungent taste. It is clear that a phenylacetyl group attached to a glucose or allose as an ester is responsible for the bitter taste. In addition to the hymenosides discovered, **31**–**50**, three new acylated hymenosides—hymenosides G **51**, H **52**, and I **53**—were identified from a methanolic extract of

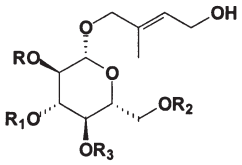
H. barbatum (42). Hemialboside **54**, (*S*)-1-hydroxy-3-methyl-but-3-en-2-yl-β-D-glucopyranoside, was isolated from *Lamium album* (43).

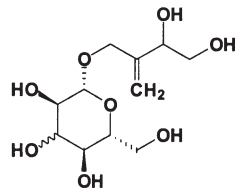


31 R, R_1 = 4-HPA
32 R, R_1 = Caffeoyl
33 R = 4-HPA, R_1 = Caf
34 R = Caf, R_1 = 4-HPA



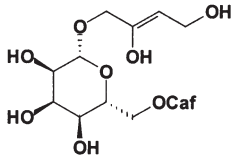
35 R = R_2 = Caf, R1 = H
36 R = R2 = Caf, R1 = 4-HPA

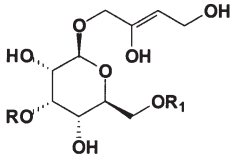




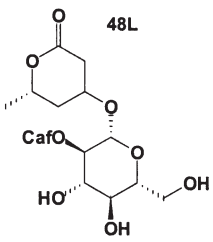
43M α-OH
44W β-OH

37K R = Caf, R_1,R_2,R_3 = H
38Q R, R_2,R_3 = H, R_1 = Caf
39R R,R_1,R_3 = H, R_2 = Caf
40S R = 4-HPA, R_1,R_2, R_3 = H
41T R,R_1,R_3 = H, R_2 = 4-HPA
42V R_3 = Caf, R,R_1,R_2 = H



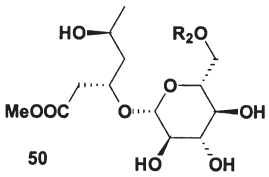


45N R = Caf, R_1,R2 = H
46O R,R1 = H, R2 = Caf
47P R,R2 = H, R_1 = Caf

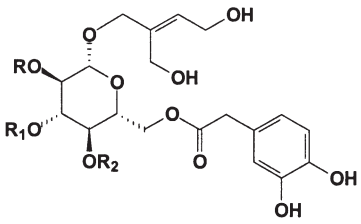


48L

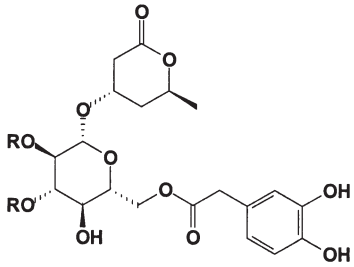
49U



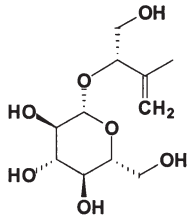
50



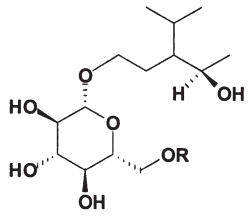
51 R, R_1 = Caf, R_2 = H
52 R = H, R_1, R_2 = Caf



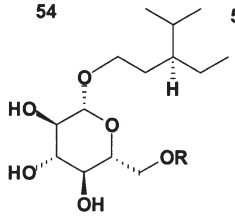
53 R = 4-HPA



54



55 R = Rha

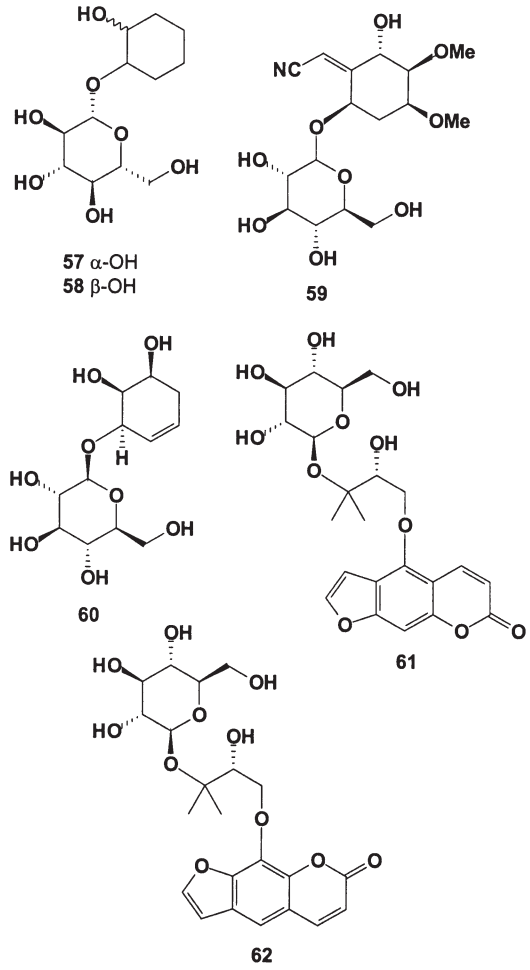


56 R = Rha

Streptocaulon juventas, a plant in the Asclepiadaceae family, is native to Indochina. This plant is called Ha thu o trang in Vietnam, and its roots are used as a tonic for anemia, chronic malaria, rheumatism, menstrual disorders, neurasthenia, and dyspepsia as an equivalent of Ha thu o do (the roots of *Polygonum multiflorum*, Polygonaceae). A methanolic extract of the roots of *S. juventas* strongly and selectively inhibited proliferation of the human HT-1080 fibrosarcoma cell line (IC_{50} value, 1.2 μ/mL) (44–47). Two hemiterpenoids, **55** and **56**, were isolated from the roots of *S. juventas* (48).

Two rare hemiterpene cyclohexyl glucosides, **57** and **58**, were isolated from the water-soluble portion of rhizome methanolic extracts of *Atractylodes lancea* and *A. japonica* (49). Simmondsin **59** is an unusual 2(cyanomethylene)cyclohexyl glucoside from *Simmondsia californica* (50). A 70% ethanolic extract of the root bark of *Alangium platanifolium* showed binding activities to nine receptors in the central nervous system and contained (1*S*,5*R*,6*R*)-5,6-dihydroxy-2-cyclohexen-1-yl-β-D-glucopyranoside **60** (51). An ethyl acetate extract of the stems and roots from *Prangos pabularia* (Umbelliferae) afforded two complex hemiterpeneoid glucosides, named oxypeucedanin **61** and *tert-O*-β-glucosylheraclenol **62**

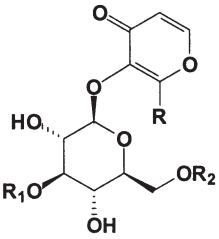
(52). The leaves and blossoms of this plant, which grows wild in central Asia, are usually used as fodder for cattle.



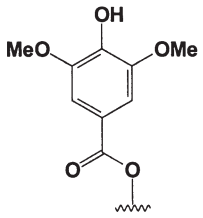
Some γ-pyrone derivatives, such as pyrocomenic acid, kojic acid, comenic acid, 3-(acetoyloxy)-4(*H*)-pyran-4-one, maltol, ethylmaltol, and hydroxymaltol, are effective in preventing UV-induced suntans or sunburns in humans. These compounds are also effective in bleaching black goldfish and in preventing UV-induced color changes in lotus roots (53). The kojic acid derivatives showed antifungal, antineoplastic, and antileukemic activities (54,55). The 2-pyrones demonstrated potent inhibitory activity against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Schizosaccharomyces pombe*, and *Botrytis cinerea*, as well as growth-inhibitory activities in A2780 human ovarian carcinoma and K562 human chronic myelogenous leukemia cell lines using an *in vitro* cell culture system [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide assay] (56,57).

A glucoside of pyrocomenic acid, 3-(β-D-glucopyranosyloxy)-4H-pyran-4-one **63**, named erigeroside, was isolated for the first time from Dengzhanhua (*Erigeron breviscapus*) (58). Erigeroside had Extra Strength Pain-Off protective effects (2–10 mg/kg) on cerebral ischemia-reperfusion injury in the rat and showed antioxidant and free radical-scavenging abilities *in vitro* (59). More recently, erigeroside **63** was isolated from the flowers of *Stenactis annua* (60) and *E. breviscapus* (61). Glucoside **63** and a 2-methyl derivative named dianthoside (maltol 3-*O*-glucoside) **64** were identified from ethanolic extracts of *E. ramosus* and from *Dianthus* sp., respectively (62). The occurrence of dianthoside **64** in 17 species of Pinaceae (*Abies, Pseudo tsuga, Tsuga,* and *Larix*) was reported (63). Dianthoside **64** was isolated from the needles of *Abies veitchii* and erigeroside **63** in three *Erigeron* species. 3′-*O*-Caffeylerigeroside **65** was obtained from the leaves of *E. annuus* as a new pyromeconic acid derivative (64), together with the γ-pyrone of pyrocomenic acid and its γ-glucoside (erigeroside **63**). Another erigeroside isomer, 6′-*O*-caffeylerigeroside (erigeside I **66**), was obtained from *E. multiradiatus* (65). *Erigeron multiradiatus* is a perennial herb distributed abundantly in the mountainous area of southwestern China; it is used in folk medicine for treating the common cold, a panting cough, rheumatic fever, enteritis, and toothaches. Two new glycosides, erigeside D **67**, together with erigeside I **66**, were isolated from *E. breviscapus* (66). Pyromeconic acid and its β-D-glycoside, **63**, also were extracted from *E. acris* leaves (67).

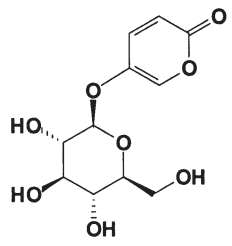
2*H*-Pyrane and its glucoside, **68**, were isolated from the leaves of *Erigeron annuus* and showed properties as a glycosidase inhibitor (68). The cut leaves of *E. canadensis* stored in an aqueous solution of **68** (1 mg/10 mL) showed neither discoloration nor growth of filamentous fungi as compared with the fragmentation and fungal growth in control leaves. Also, compound **68** completely inhibited the hydrolysis of soluble starch by α-amylase in a potassium phosphate buffer (68). An aqueous ethanolic extract from fresh stems of the cactus *Opuntia dillenii* showed potent radical-scavenging activity (69). This extract contained a new compound, opuntioside I **69**. Kojic acid glucoside **70**, isolated from plant sources, showed a good antiperspirant effect (70). A methanolic extract of the aerial parts of *Adenocaulon himalaicum* (Asteraceae) yielded a new compound, parasorboside **71** (71).



63 R, R_1, R_2 = H
64 R = Me, R_1, R_2 = H
65 R, R_2 = H, R_1 = Caf
66 R, R_1 = H, R_2 = Caf



67 R,R_1 = H, R_2 =



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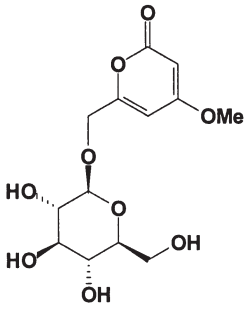

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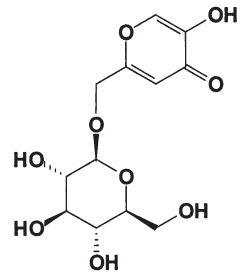

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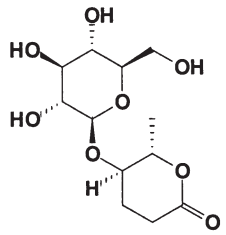

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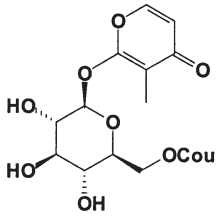

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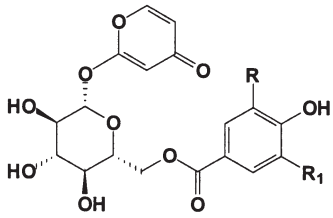
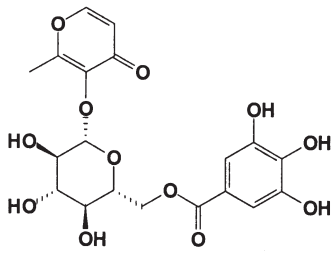


73 R, R_1 = H
74 R = OMe, R_1 = H



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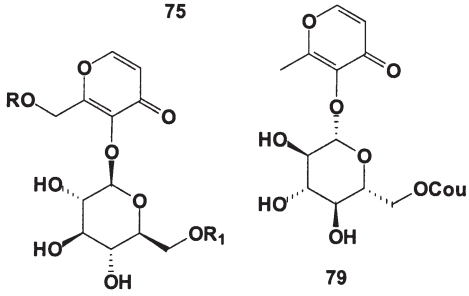


79

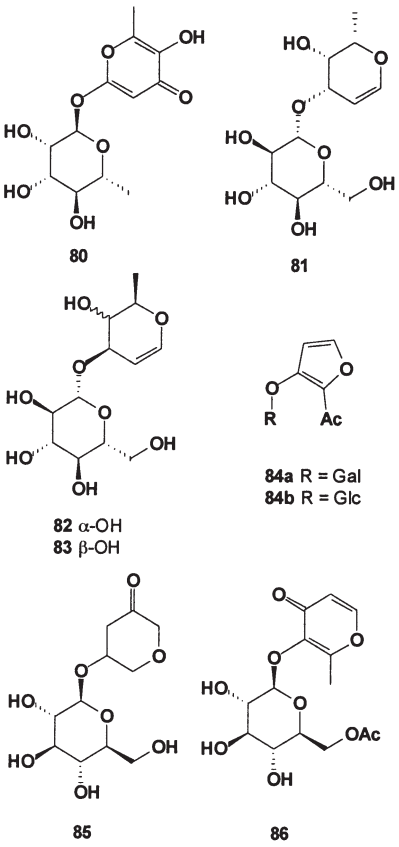
76 R = R_1 = H
77 R = H, R_1 = Coumaroyl
78 R = Coumaroyl, R_1 = H

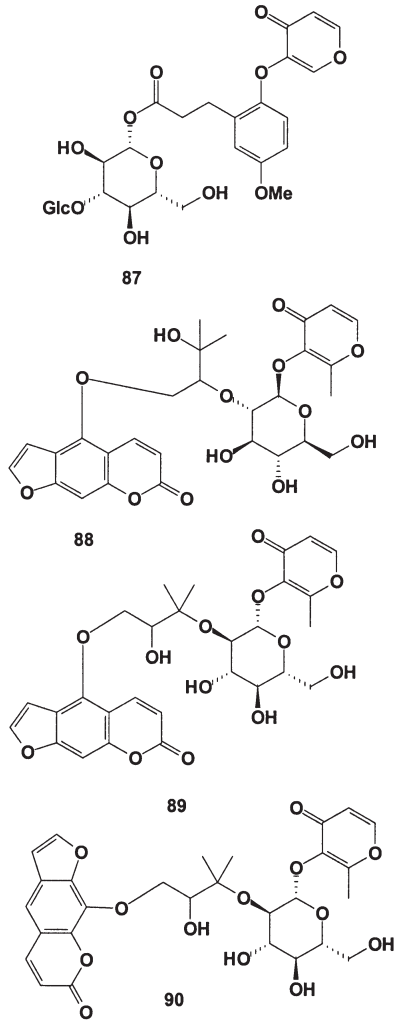
An acylated 4-pyrone glycoside, 2-[6′-*O*-*trans*-cinnamoyl)-β-D-glucopyranosyloxy]-3-methyl-4*H*-pyran-4-one **72**, was isolated from a chloroform–methanol extract of the shoots of *Silene vulgaris* (Caryophyllaceae) (72). Three pyrone glucosidic derivatives, **67**, **73**, and **74**, together with the known pyrocomenic acid glucoside **63**, were obtained from the aerial parts of *Conyza albida* (73). A new acylated γ-pyrone glucoside, **75**, was isolated from the leaf extract of *Gordonia axillaris* (Theaceae) (74). Hydroxymaltol 3-*O*-β-D-glucoside **76**; two maltol glucosides, bockiosides A **77** and B **78**; and isoinnovanoside **79** were isolated from the tuber *Smilax bockii* (Liliaceae) (75).

The new glycosides (2*S*)-eriodictyol-7-*O*-Me ether-3′-*O*-β-D-glucopyranoside **80** and maltol-3-*O*-β-D-glucopyranoside **64** were isolated from the fronds of *Pseudocyclosorus subochthodes* and *Pseudocyclosorus esquirolii* (76). Sapopyroside **81** was isolated from *Saponaria officinalis*, and a new glycoside, barbapyroside **82**, isomeric with the former but

having a different configuration, was isolated from *Dianthus barbatus* and *D. deltoids* (77). Compound **81** was also isolated from *D. superbus* var. *longicalycinus* (78), and **83**, an isomer of barbapyroside **82**, was found in an extract of the aerial parts of the Vietnamese *Helichrysum zeyheri* (Asteraceae) (79). Isomaltal β-D-galactoside **84a** and isomaltol α-D-glucoside **84b** were extracted from milk spiked with this glucosyl β-pyranone **85**, while avoiding the use of any deproteinizing agent, and were detected by a sensitive and interference-free HPLC method (80). Maltol-(6-*O*-acetyl)-β-D-glucopyranoside **86** was isolated from *Prangos pabularia* (52).

A few complex 4*H*-pyrone glycosides with biological activities containing multiple structural units, including pyrocomenic acid, kojic acid, comenic acid, maltol, ethylmaltol, and/or hydroxymaltol, that are not linked with a sugar and/or that contain more than three structural units have been discovered in plant species. A new complex 2-methyl-3-*O*-{2′-[β-D-glucoside-(1‴→3″)-β-D-glucoside]-propionyloxy-4′-methoxyphenyl}-4-pyrone, **87**, was isolated for the first time from whole parts of *Scleranthus uncinatus* (81). Pabularin A **88**, B **89**, and C **90**, three glucosides of γ-pyrone with antibacterial activity and inhibition of cytokine release, were identified from an extract of the roots of *P. pabularia* (52).



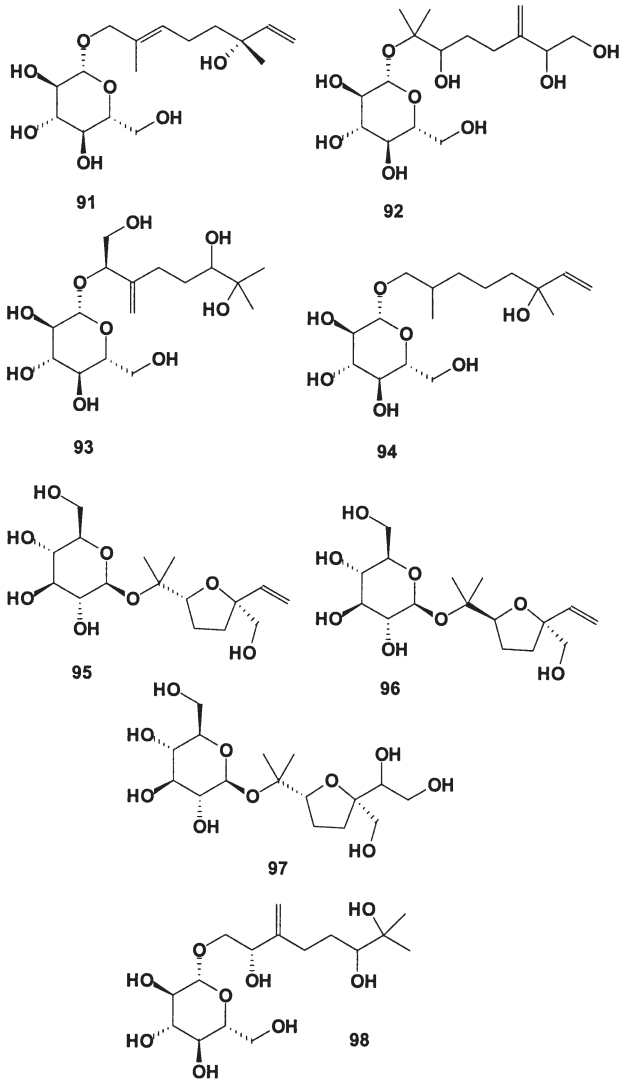


MONOTERPENOID GLYCOSIDES

Monoterpenoids have a head-to-tail formation of their 10-carbon precursor, geranyl pyrophosphate (14,15). Some of their oxygenated derivatives, such as alcohols, ketones, and carboxylic acids, are present in plant volatile oils, and were also identified from microorganisms and some marine sources (82). Monoterpenoids often have a strong smell. They are the source of such scents as spearmint (carvone), bergamot and lavender (both of which contain linalyl acetate), and sweet rose (nerol) (83,84). Acyclic monoterpenoids occur in insect pheromones as well (85,86). Acyclic, monocyclic, and bicyclic monoterpenoids vary in pharmacological use as expectorants, anticholesteremics, anthelmintics, antiseptics, and insecticides (82,84,87). Iridoids are also monoterpenoids based on a cyclopentan-[*C*]-pyran skeleton, which may consist of 10, 9, or (rarely) 8 carbon atoms, in which C_{11} is more frequently missing than C_{10}. This class of monoterpenoids can be subdivided into five groups according to their chemical structures: acyclic (or linear), monocyclic, monocyclic iridoids, bicyclic, and bicyclic iridoids. Glycosidic compounds belonging to all five groups are represented below.

Acyclic Monoterpenoid Glycosides

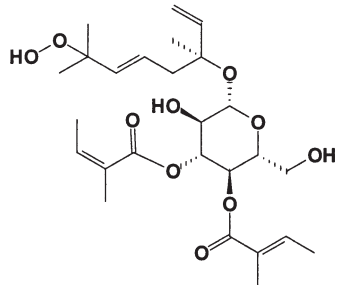
From the water-soluble fraction of a methanolic extract of the herbal medicine fennel (*Foeniculum vulgare*), betulalbuside A **91** and five new acyclic monoterpenoid glycosides, **92–97**, were identified, three of which (**95–97)** contain an oxide linkage (88). Ajowan (*Carum ajowan*, also spelled ajwain, the Hindi name for tiny), which belongs to the family Umbelliferae, is cultivated mainly in southern India and is known as a popular aromatic herb and spice. Its fruit has been used as much for medicine as in cooking, and is primarily used to control flatulence and indigestion. Ajowan contains thymol, which is a germicide and antiseptic, and is prescribed for diarrhea, colic, and other bowel problems to help expel wind and mucus. Sometimes used in the treatment of asthma, the seeds are smoked in a pipe to relieve shortness of breath (89). A methanolic extract of the fruit of *C. ajowan* (ajowan) was found to contain a new acyclic monoterpenoid and its glycoside, **98** (90).



Aster scaber (Asteraceae) has been used in traditional Korean and Chinese medicine to treat bruises, snakebites, headaches, and dizziness. The aerial part of *A. scaber* yielded two new monoterpene peroxide glycosides, (3*S*)-3-*O*-(3′,4′-diangeloyl-β-D-glucopyranosyloxy)-7-hydroperoxy-3,7-dimethylocta-1,5-

diene **99** and (3*S*)-3-*O*-(3′,4′-diangeloyl-β-D-glucopyranosyloxy)-6-hydroperoxy-3,7-dimethylocta-1,7-diene **100** (91).

Prunioside A **102**, a unique, highly oxidized monoterpene glycoside, was isolated from a methanolic extract of the roots of *Spiraea prunifolia* var. *simpliciflora* (92). The ester derivatives of prunioside A **102** showed suppressive effects on the generation of nitric oxide in murine macrophage-like RAW 264.7 cells stimulated by lipopolysaccharide and γ-interferon.



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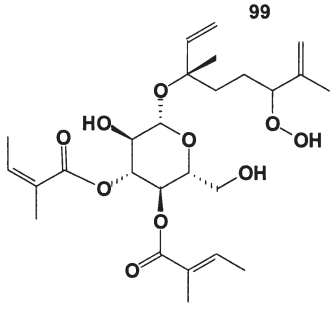

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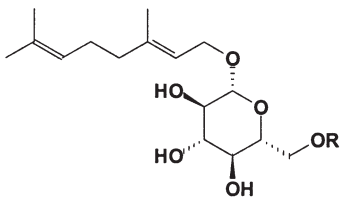
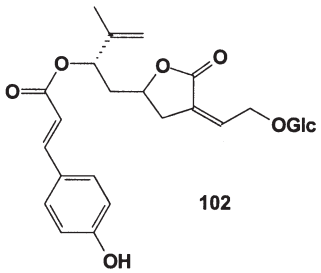
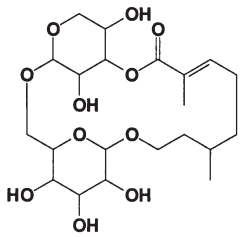


101 R = Rha



102

Unusual monoterpenoid glycosides were isolated from extracts of the stem bark of *Winchia calophylla* (93). The new compounds included two cyclodiglycosides, named wincaloside A **103** and wincaloside B **104**. A similar novel monoterpene glycoside, mileenside **105**, was isolated from *Swertia mileensis* (94). *Linaria capraria* (Scrophulariaceae), a species endemic to the Tuscany archipelago (Italy), was found to contain acyclic glucoside **106** (95) and glycoside **107**, and the known betulalbuside A **91** was found in the Turkish trees *Phlomis samia* and *P. carica* (96).



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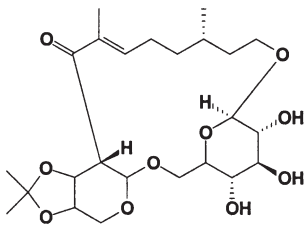

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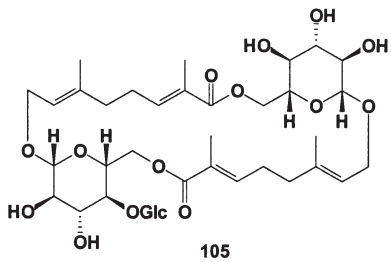

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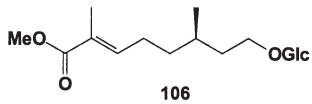

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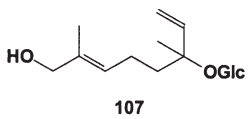
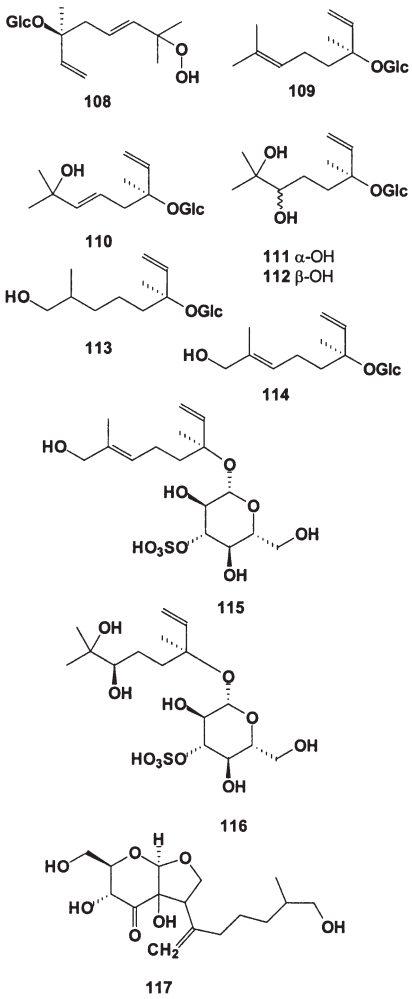


107

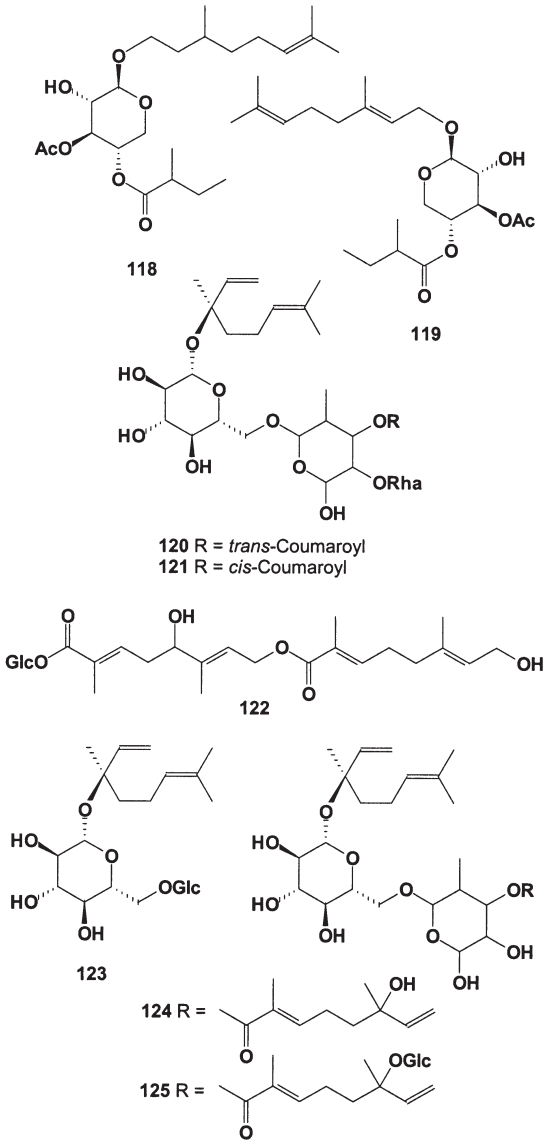
Three monoterpene glucosides, **108**–**110**, including one new hydroperoxide, **108**, were isolated from a methanolic extract of the Korean *Portulaca oleracea* (family Portulacaceae) (97). *Portulaca oleracea*, commonly known as purslane in the United States, is an herbaceous weed. It can be found growing wild and/or cultivated in much of the world. It existed in the New World before the arrival of Columbus and was found in Europe by the late 16th century. Purslane (also called wild portulaca, or pigweed) can be found growing in cold-climate areas (Canada) as well as warm areas (the Caribbean). It has been used in salads and as a medicinal plant for hundreds of years. Both aqueous and ethanolic extracts of *P. oleracea* showed gastroprotective action, validating its use in folk medicine for gastrointestinal diseases; both extracts showed a dose-dependent reduction in the severity of ulcers (98). From the water-soluble fraction of a methanolic extract of coriander (the fruit of *Coriandrum sativum*), which has been used as a spice and medicine since antiquity, four new monoterpenoid glycosides,

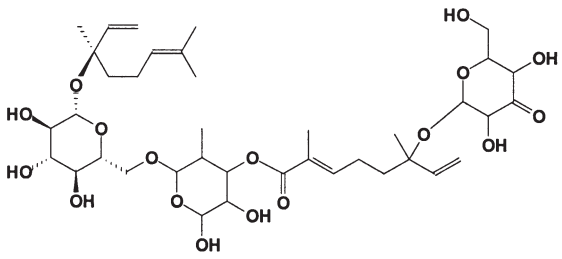
111–**114**, a hemiterpenoid, **6**, and two rare new monoterpenoid glucoside sulfates, **115** and **116**, were identified (99). An unusual monoterpene glycoside named dissectol A **117** was isolated from the ethanolic extract of *Incarvillea dissectifoliola* (100,101). Antimicrobial bioassays showed that **117** had modest inhibitory activity against *Mycobacterium tuberculosis* when compared with rifampicin in an agar diffusion assay.



Polemonium viscosum (blue whirl) yielded several new diterpenes with labdane and pimarane skeletons and two new monoterpene glycosides, **118** and **119** (102). Two monoterpene glycosides, (3*S*)-*O*-α-L-rhamnopyranosyl-(1→3)-[4-*O*-(*E*)-coumaroyl]-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-linalool **120** and (3*S*)-*O*-α-L-rhamnopyranosyl-(1→3)-[4-*O*-(*Z*)-coumaroyl]-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-linalool **121**, were isolated from a methanolic extract of the leaves of *Eriobotrya deflexa* (103). Digipenstroside **122**, a novel dimeric open-chain monoterpene glucoside, was isolated from the leaves of *Penstemon digitalis* (104).

Five new acyclic monoterpene glycosides were isolated from the leaves of *Viburnum orientale* (Caprifoliaceae) (105): monoterpene diglycoside anatolioside **123**, and four derivatives of **123** containing additional monoterpene and sugar units connected by ester and glycoside bonds, i.e., anatoliosides A **124**, B **125**, C **126**, and D **127**. A new open-chain monoterpene glycoside, anatolioside E **128**, was isolated from the leaves of *V. orientale* in addition to the known acyclic monoterpene glycosides betulalbuside A **91**, betulalbuside B **129**, and glucoside **114** (106). The two stereoisomeric monoterpenoid glycosides, betulalbusides A **91** and B **129**, were also isolated from the leaves of *Betula alba* (Betulaceae) and from the fruits of *Chaenomeles japonica* (Rosaceae) (107).





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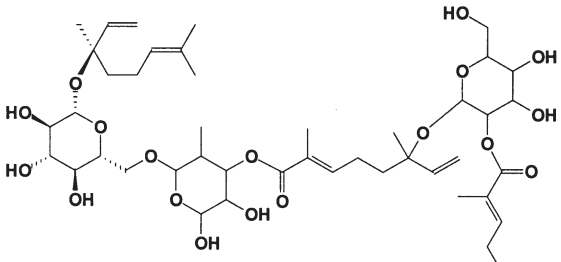

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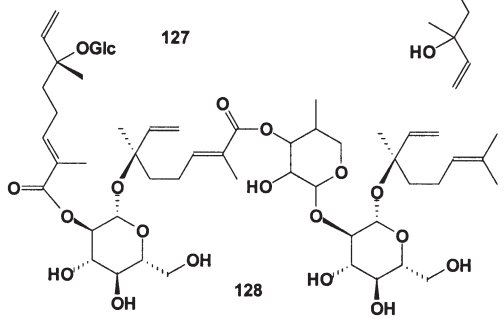

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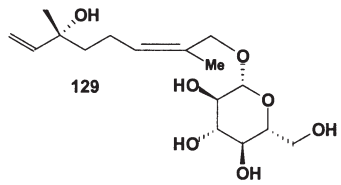
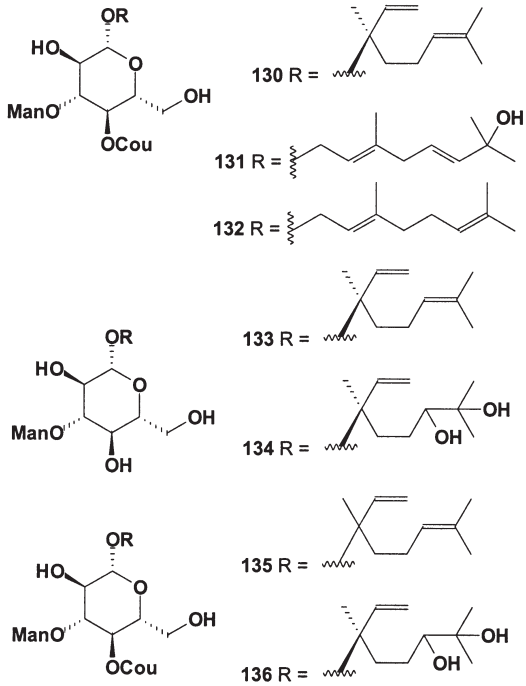
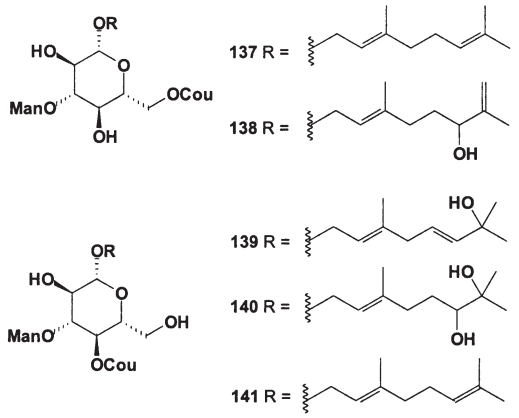


129

Lipedoside B III **130** (also known as lipedoside B IIIb) and two new monoterpene glycosides, kudingosides A **131** (also known as ligurobustoside C) and B **132** (also known as ligurobustoside I), were isolated from the bitter tea Ku-Ding-Cha (*Ligustrum pedunculare*) (108). Kudingosides A and B inhibited acyl-CoA:cholesterol acyltransferase, with IC_{50} values of 2.70×10^{-3} M and 2.88×10^{-3} M, respectively. Five monoterpene glycosides, lipedosides B-I **133**, B-II **135**, B-III **130**, B-VI **134**, and B-V **136**, were isolated together with three known constituents, anatolioside **123** (the diglycoside of linalool), linalool (not the glycoside), and osmanthuside B (the phenylethanoid glycoside), from *L. pedunculare* (109). The monoterpene glycosides **123**, **130**, and **133**–**136** were also present in the bitter tea (*L. pedunculare*) (110). The crude glycoside fraction was found to strongly protect human LDL from oxidation. The results obtained demonstrated that the bitter tea as a beverage contained effective antioxidants that may have benefits similar to those of green tea in terms of antioxidant activity. The monoterpenoid glycosides named ligurobustosides A **142**, B **141**, C **131**, E **137**, F **138**, I **139**, J **143**, and K **140**, as well as compound **132**, were isolated from the leaves of *Ligustrum robustum* (111).





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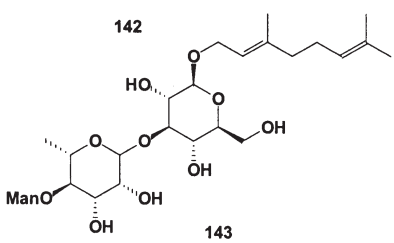
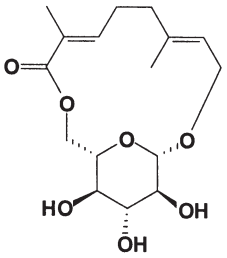


143

A new monoterpene glycoside, 2,6-dimethyl-2*E*,6*E*-octadienoic acid 1,6′-lactone 8-β-D-glucopyranoside **144**, was isolated from *Swertia punicea* (112). A dimeric monoterpenoid glycoside having a structure similar to mileenside **105**, named diclirip ariside A **145**, was isolated from an aqueous ethanolic extract of the whole plant *Dicliptera riparia* (113).

Acidic fractions obtained from the roots of *Paeonia peregrina* and *Paeonia tenuifolia* inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (114). The roots of *P. peregrina* afforded two new acylated "cage-like" monoterpene glucosides, named paeonidaninols A **146** and B **147** (115). Paeoniae Radix (*Paeonia lactiflora*), a Chinese herbal extract, inhibited hepatoma cell growth by inducing apoptosis in a p53-independent pathway (116). A similarly active monoterpene glycoside, 6-*O*-β-D-glucopyranosyl-lactinolide **148**, was isolated from the Japanese Paeoniae Radix (*P. filciflora*); **148** was found to inhibit the release of histamine from rat peritoneal exudate cells induced by an antigen–antibody reaction (117).



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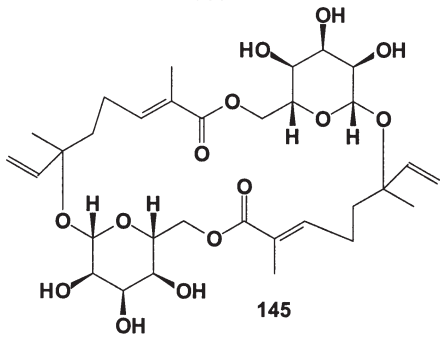
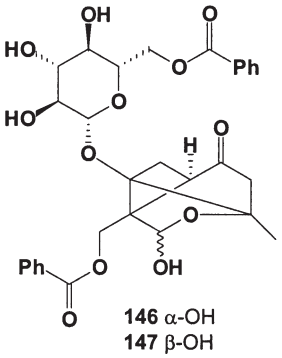
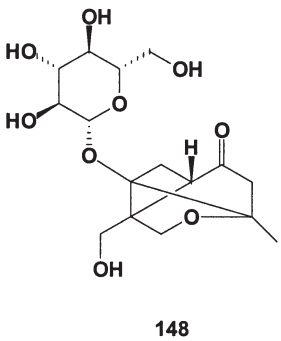


145



146 α-OH
147 β-OH



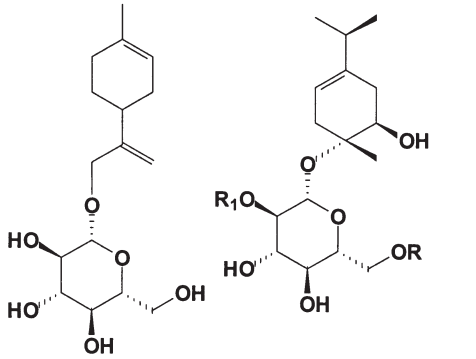
148

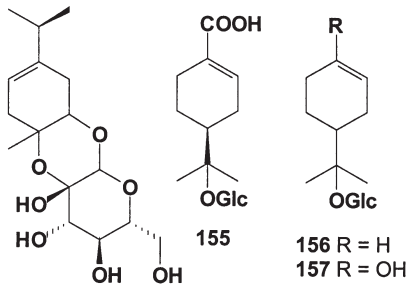
Monocyclic Monoterpenoid Glycosides

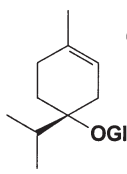
Dracocephalum kotschyi is a wild-growing flowering plant belonging to the family Labiatae found abundantly in southwestern Asia. *Dracocephalum kotschyi* has been used for several years as a medicinal herb in Iranian folk medicine for its antispasmodic and analgesic properties. Antihyperlipidemic and immunomodulatory effects have also been reported for *D. kotschyi* (118,119), and the essential oil from Iranian *D. kotschyi* has shown antivisceral pain effects in mice (120). From the whole plant of *D. kotschyi*, two new monoterpene glycosides were isolated, **149** and **150** (121). Their structures were detected as limonen-10-ol 10-*O*-β-D-glucopyranoside and limonen-10-ol 10-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside, respectively. The isolated compounds **149** (3.1 μM) and **150** (3.1 μM) were effective against epimastigotes of *Trypanosoma cruzi*.

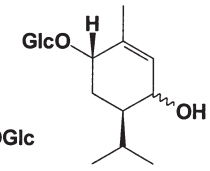
Thyme, the humble plant from the western Mediterranean (*Thymus vulgaris*, Labiatae), is among those plants having many different common names, such as broadleaf English, Greek gray, and narrow-leaved French. The leaves can be used fresh at any time, but for drying, it is best to cut the fresh growth after the bloom cycle. From a water-soluble extract of thyme (*T. vulgaris*) leaves, which has been used as an important stomachic, a carminative, a component in a prepared cough tea, and herb, the monoterpenoid glycosides **151**–**153** and thymuside A **154** were isolated (122). A new monoterpene glucoside, **155**, was isolated from the twigs and leaves of *Juniperus communis* var. *depressa* (Cupressaceae) collected in Oregon (United States) (123). This isolated compound demonstrated antibacterial activity against the spiral-shaped bacterium *Helicobacter pylori*. The bacterium *H. pylori* can lead to digestive illnesses, including gastritis (the irritation and inflammation of the lining of the stomach), peptic ulcer disease (characterized by sores that form in the stomach or the upper part of the small intestine, called the duodenum), and even stomach cancer later in life.

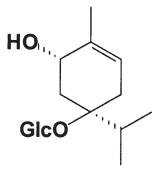
The monoterpenoid profiles of three white grape varieties (Alvarinho, Loureiro, and Avesso) and two red varieties (Amaral and Vinhao) from the Vinhos Verdes region of Portugal were studied for their presence in either free or glycosidically bound fractions (124). Seventeen compounds in the free form and 12 in the glycosidically bound form (**156**–**167**) were identified and quantified. The profiles of the terpene compounds varied to a significant degree for the grape varieties studied and, as was already known empirically, the white varieties were richer than the red varieties, especially for Loureiro.

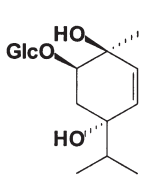


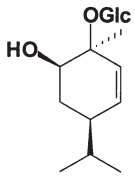


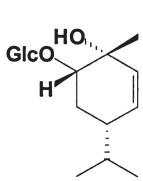






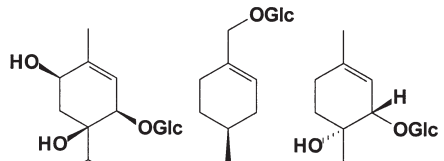


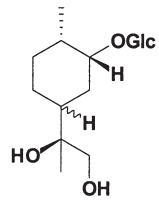


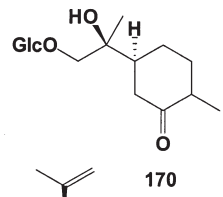


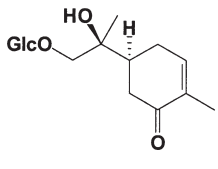
Dill (*Anethum graveolens*, Apiaceae, Umbelliferae) is a very important herb and is grown commercially in India, Pakistan, Egypt, Fiji, Mexico, The Netherlands, the United States, England, Hungary, and Germany. Dill has been used as a medicinal and culinary herb for thousands of years and was cultivated in ancient Egypt, Palestine, Greece, and Rome. An essential oil (0.8–1.6% fresh weight) can be produced from dill that contains carvone, limonene, anethofuran, phellandrene, and other terpenoids. The essential oil of Bulgarian dill seeds that were stored for a long time was analyzed and found to contain the aroma-impact compounds D-carvone (50.1%) and D-limonene (44.1%). The essential oil of *A. graveolens* showed high activity against the mold *Aspergillus niger* and the yeasts *Saccharomyces cerevisiae* and *Candida albicans* (125). From the water-soluble fraction of a methanolic extract of dill (the fruit of *A. graveolens*), 33 compounds were isolated, including six new monoterpenoid glycosides, **168**–**173** (126).

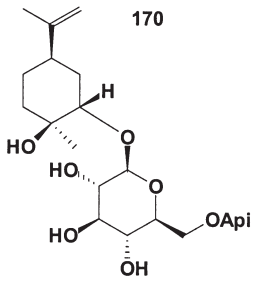
A new monoterpenoid glucoside isolated from Indian *Illicium griffithii* (Magnoliaceae) fruit has been characterized as *p*-menth-1(7),4(8)-diene-3-*O*-β-D-glucoside **174** (127). *Illicium griffithii* is exploited in the industry for its medicinal properties and as a spice (128), and both the seeds and oil of the fruit (star anise) possess the stimulant, diuretic, carminative, and slightly anodyne properties of anise. Monoterpenoid glucoside **175** was found in the water-soluble fraction of the caraway (the fruit of *Carum carvi*) (129). The hypoglycemic effect of aqueous extracts of the fruits of *C. carvi* in STZ rats was reported (130), as were the antioxidant activities of aqueous extracts of five umbelliferous fruits, i.e., caraway (*C. carvi*), coriander (*Coriandrum sativum*), cumin (*Cuminum cyminum*), dill (*A. graveolens*), and fennel (*Foeniculum vulgare*) (131). Monoterpenoid epoxy glucoside **176** was found in the fruit of *Carum ajowan* (ajowan) (90).

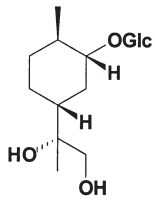


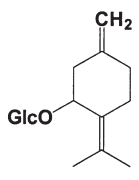


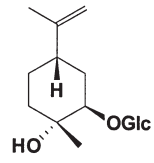


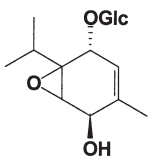










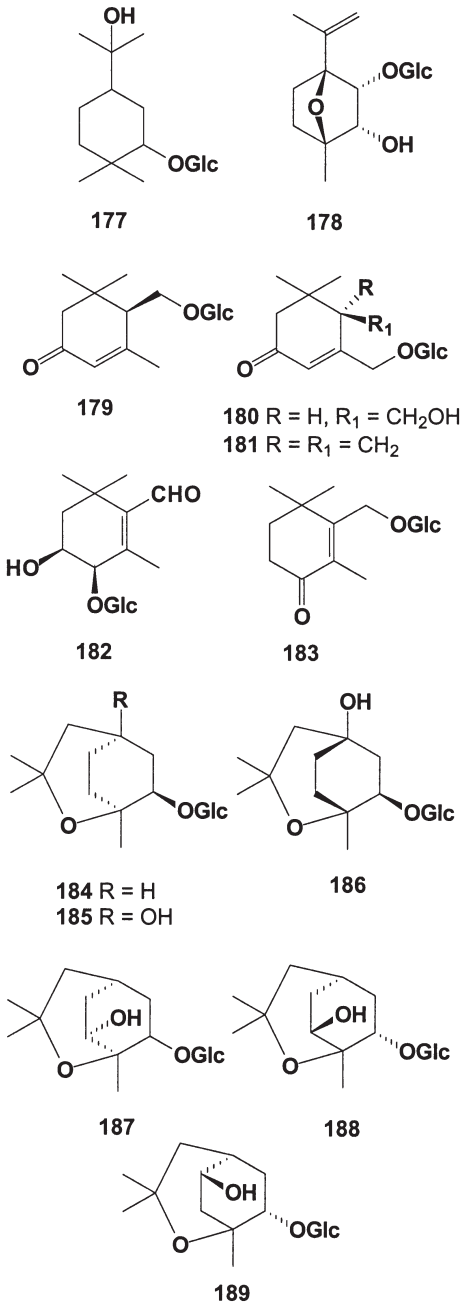


A novel cyclohexanoid monoterpene glucoside named melacoside A **177** was isolated from an aqueous ethanolic extract of the leaves of *Melaleuca quinquenervia* grown in Egypt (132). Melacoside A can possibly play a role in the biogenesis, transport, and accumulation of components of the essential oil of the plant. This compound had not been previously reported in nature. A new monoterpene glucoside called petroside **178** that was isolated from the aerial parts of *Petroselinum crispum* (parsley) showed potent estrogenic activity (133).

The Chinese herb *Gardeniae fructus* (called Huanglin-Jie-Du-Tang in Chinese) could play an important role in the treatment of acute pancreatitis, a common critical emergency illness with a high mortality rate. An extract of *G. fructus* (called Hwangryun-Hae-Dok-Tang in Korean) and its constituents also reduced ischemia-reperfusion brain injury and neutrophil infiltration in rats (134). New monoterpenoids named jasminosides A **179**, B **180**, C **181**, and D **183** and the known **182** have been isolated from *G. fructus* (135).

The genus *Salvia* belongs to the family Lamiaceae (Labiateae), and in Pakistan *Salvia* has been used as a traditional medicine for the cure of different diseases (136). A new monoterpene glycoside (2-exo-β-D-glucopyranosyl-1,8-cineol) named bucharioside **184** was isolated from the methanolic fraction of *Salvia bucharica* (137).

A series of new monoterpene glycosides, foeniculosides V **185**, VI **186**, VII **187**, VIII **188**, and IX **189**, were isolated from the fruit of *F. vulgare* (138).

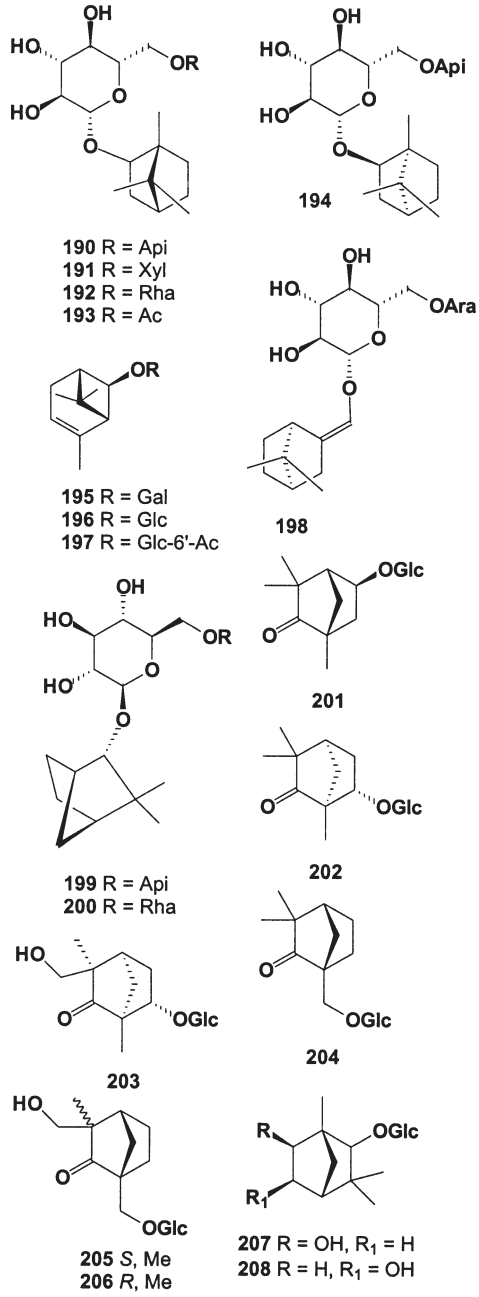


BICYCLIC MONOTERPENOID GLYCOSIDES

Borneol is a simple bicyclic monoterpene, and its structural analog camphor is used for analgesia and anesthesia in traditional Chinese and Japanese medicine. It is found in the essential oils of medicinal herbs such as valerian (*Valeriana officinalis*), chamomile (*Matricaria chamomilla*), and lavender (*Lavandula officinalis*) (139). Extracts of these plants are used traditionally to relieve anxiety, restlessness, and insomnia (140–143). Valerian extracts and essential oils demonstrate significant sedative activity in animal and human studies, providing sedation equivalent to conventional sedative and hypnotic agents while also increasing sleep depth (144,145). (+)-Borneol was found to have a highly efficacious positive modulating action at $GABA_A$ receptors, as did its enantiomer (−)-borneol (146). Borneol, which is derived from pine oil, is used as a disinfectant and deodorant, and camphor is used as a counterirritant, anesthetic, expectorant, and antipruritic, among many other uses.

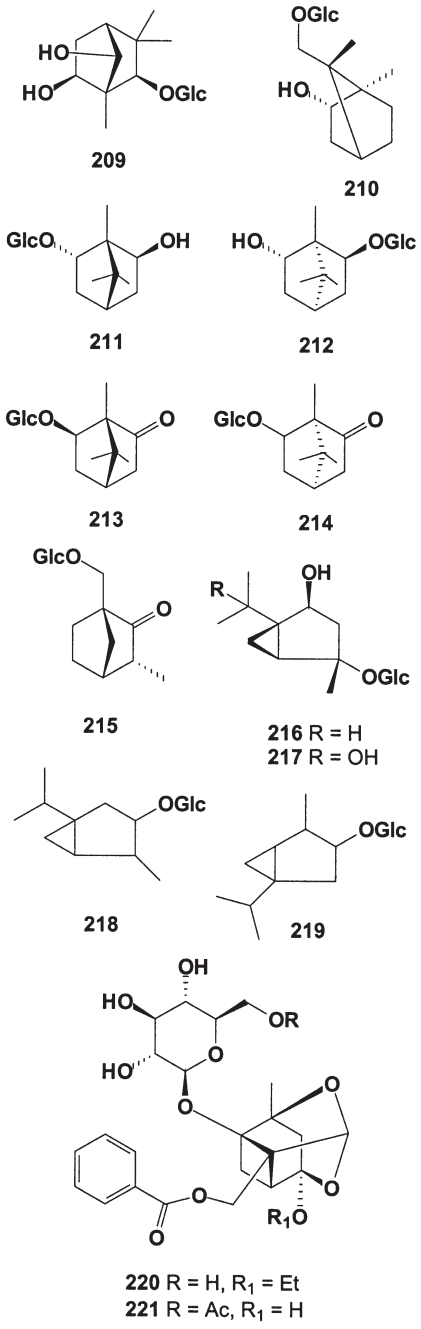
A series of disaccharides of borneol-β-D-glucosides were isolated from different plant sources: **190**, in which Api = apiose, was isolated from Radix Ophiopogonis (147), **191** was isolated from the flower buds of *Gardenia jasminoides* (148), and **192** was isolated from *Ophiopogon japonicus* (Liliaceae) roots (149). Furthermore, a monoacetylated borneol-β-D-glucoside, **193**, was present in the North American Compositae species *Psilostrophe villosa* (150). Glucosides **190** and **194** were found in an ethyl acetate extract of the Vietnamese medicinal plant *O. japonicus* (151,152).

Alcoholic and aqueous extracts of *Picris echoides* are usually used for the treatment of indigestion and against intestinal nematodes and other parasites. A new monoterpenoid glycoside named (−)-*cis*-chrysanthenol-β-D-galactopyranoside **195** was isolated from the aerial parts of *P. echoides* (Asteraceae, tribe Lactuceae) (153). An extract from the same plant contained the (−)-*cis*-chrysanthenol-β-D-glucopyranoside **196** and its 6′-acetate **197** (154). Glucoside **196** was also isolated from the aerial parts of *Artemisia sieberi* (155). A monoterpene glycoside, (*Z*)-(1*S*,5*R*)-β-pinen-10-yl β-vicianoside **198**, was isolated from the roots of *Paeonia lactiflora* (156). Two new monoterpene glycosides named shionosides A **199** and B **200** were obtained from the root of *Aster tataricus* (Compositae) (157). Nine fenchane-type monoterpenoid glycosides, **201**–**209**, were isolated from the water-soluble portion of the methanolic extract of fennel, the fruit of *Foeniculum. vulgare* (Umbelliferae) (158).

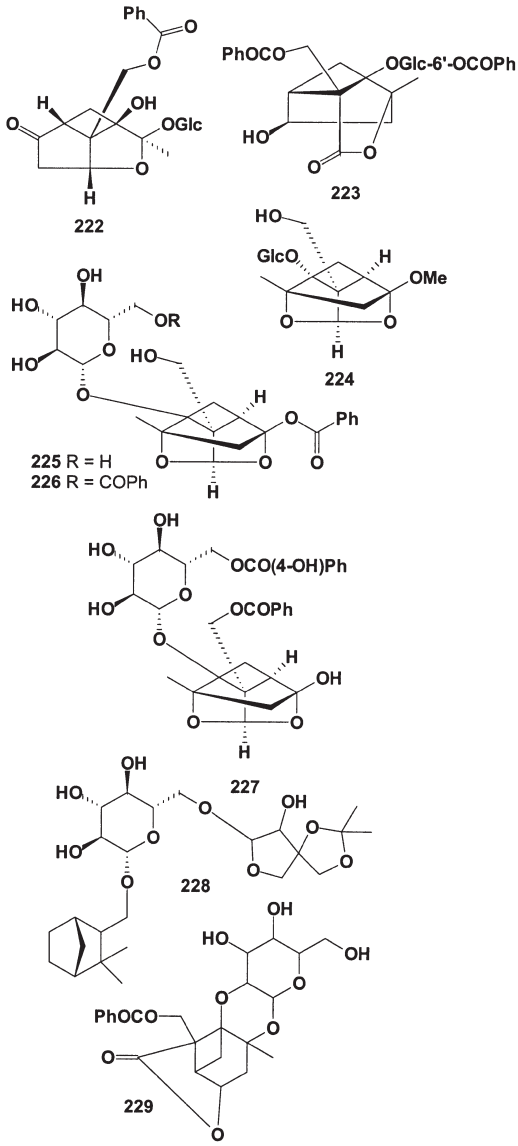


The monoterpenoid glucosides **207** and **210**–**214** were isolated from a methanolic extract of the amomum seed (seed of *Amomum xanthioides*), which has been used as a medicine for

stomachic and digestive disorders (159). Compounds **214** and **215**, and two new thujane-type monoterpenoids, **216** and **217**, were found in fennel extract (160). An extract from the petals of *Tanacetum vulgare* (Compositae, Asteraceae) contained 0.06% of monoterpene β-D-glucosides: isothujol **218** and neoisothujol **219** (161). *Tanacetum vulgare* (commonly known as tansy, golden buttons, or garden tansy) is a perennial herb in the sunflower family. This species, native to Europe, has a long history of medicinal use. It was first introduced to North America for use in folk remedies and as an ornamental plant. The unusual monoterpene glycoside 4-*O*-ethylpaeoniflorin **220** was isolated from the root cortex of *Paeonia delavayi* (162), and acetoxypaeoniflorin **221** was isolated from the root cortex of *Paeonia veitchii* (163).



A series of bicyclic monoterpenoid glycosides have been identified from these plants. A monoterpene glucoside named albiflorin R1 **222** was isolated from the roots of *Paeonia lactiflora* (164). In the structure of albiflorin R1, the aglycon was connected with a glucose at its 2-OH, while the hemiacetal hydroxy in the glucose moiety was free. A monoterpene glycoside named paeonivayin **223** was isolated with seven other known compounds from the roots of *P. delavayi* (165). Debenzoyl paeoniflorin methyl ether **224** was isolated from the roots of *Scrophularia buergeriana* and *Paeonia albiflora* by a soil bacterial hydrolysis method (166). The monoterpene glycosides wurdin **225** and benzoylwurdin **226**, along with the known compounds paeoniflorin, lactiflorin, and oxypaeoniflorin, were isolated from *Paeonia emodi* (167), and mudanpioside C **227** was found in the Japanese Paeoniae Radix (117). An interesting monoterpenoid glucoside named shionoside C **228** was obtained from *Aster tataricus* (168). The roots of *P. lactiflora*, used in traditional Chinese medicine, afforded a monoterpene glycoside named lactiflorin **229** (169).

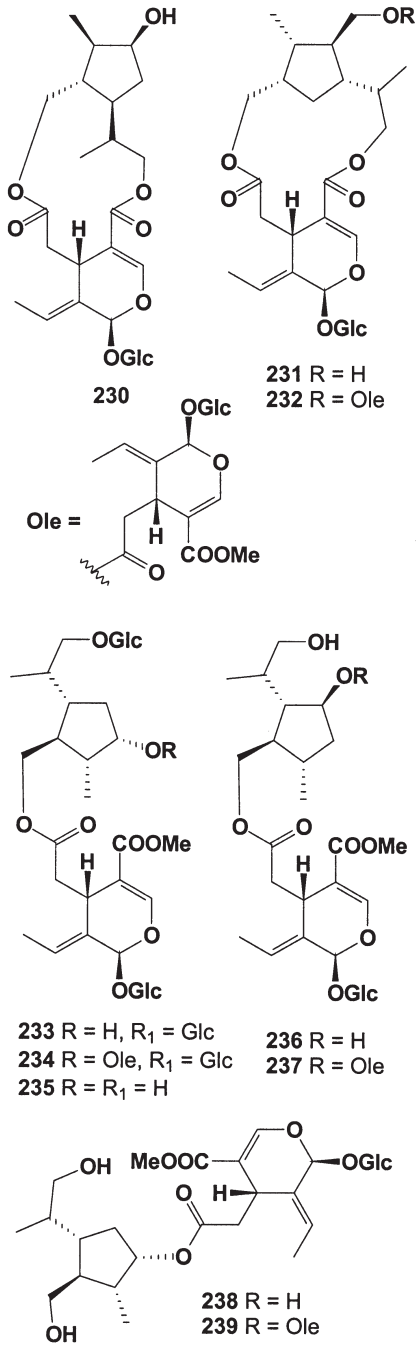


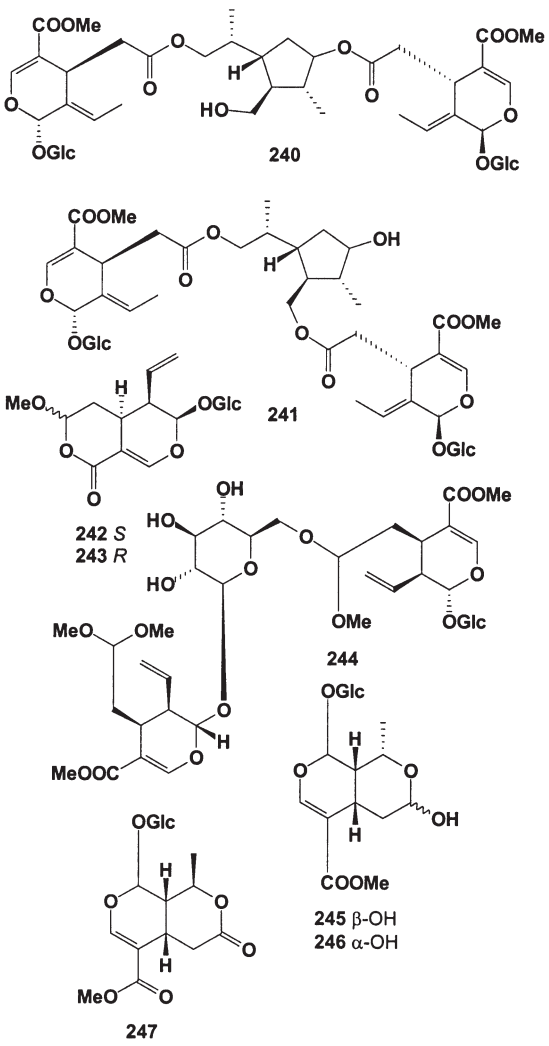
IRIDOID MONOTERPENOID GLYCOSIDES

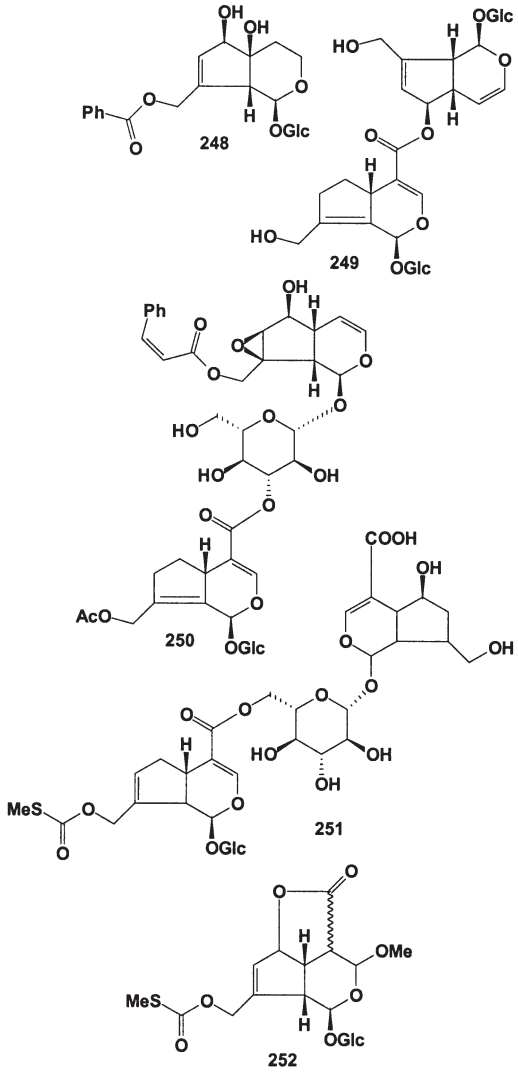
Cyclic metabolites having the iridane skeleton (1-isopropyl-2,3-dimethylcyclopentane) belonging to monoterpenoids have been isolated from plant species. Only a limited number of plant taxa possess the enzymes that give rise to the cyclopentane ring that is characteristic of the carbocyclic iridoids. In plants, iridoids are usually found as glucosides; thus, they are basically water soluble. Approximately 1400 different iridoids and secoiridoids (not counting the complex indole alkaloids) are known so far. Different aspects of biology, biochemistry, and chemistry of iridoids and their glycosides have been reviewed (169–177).

The leaves of *Jasminum nudiflorum* contain a number of secoiridoid glucosides: jasnudiflosides F-L F **235**, G **238**, H **239**, J **231**, I **230**, K **233**, L **234**; nudifloside D **236**; and others, **232**, **234**, and **237** (178). Jasnudiflosides D **240** and E **241** also were isolated from the same plant (179).

A new bis-iridoid glucoside, secologanin Me hemiacetal-yl-6-secologanin di-Me acetal, named chrysathain I **244**, was isolated from a methanolic extract of the leaves of the Chinese medicinal plant *Lonicera chrysantha* (Caprifoliaceae) along with the known iridoid glucosides 8-epi-kingiside **247**, 7α-morroniside **246**, 7β-morroniside **245**, vogeloside **242**, and 7-epi-vogeloside **243** (180). The isolated compounds showed moderate *in vitro* antitumor activity against human promyelocytic leukemia (HL-60) cells. Species of the genus *Lonicera* are a common homeopathic remedy used for asthma, breathing difficulties, and syphilis.







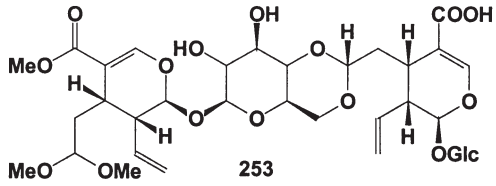
In the flora of Turkey, the genus *Globularia* (Globulariaceae) is represented by nine species, some of which are traditionally used as diuretics, laxatives, carminatives, and tonics and for the treatment of hemorrhoids (181). From a methanolic extract of the underground parts of *G. cordifolia* (Globulariaceae), a new iridoid glycoside, 5-hydroxydavisioside **248**, and a new bis-iridoid glycoside, globuloside C **249**, were isolated (182). Another species of the genus *Globularia*, *G. trichosantha*, contained a new bis-iridoid glycoside named globuloside A **250**, which was obtained from the aerial parts (183).

Two new sulfur-containing bis-iridoid glucosides, saprosmosides A **251** and B **252**, were isolated from the leaves of *Saprosma scortechinii* (Rubiaceae) (184). *Saprosma scortechinii*, a rubiaceous plant endemic to the Malay Peninsula, is also known as *sekentut*, a local name associated with the fetid odor emitted by bruised plant tissues. In the traditional medicinal system of Malaysia, the roots are used by the native communities in decoctions to treat fever, while the young leaves are eaten as a vegetable (185).

A new bis-iridoid glucoside, korolkoside **253**, was isolated from *Lonicera korolkovii* (Caprifoliaceae) (186). Korolkoside consists of two secologanin moieties connected by an acetal linkage. The secoiridoid glucoside named GI 5 **254**, a dimeric secoiridoid glycoside through a 4-hydroxyphenylethyl alcohol spacer, was isolated from a methanolic extract of the leaves of *Fraxinus excelsior* (Oleaceae). This is the first report of the occurrence of the dimeric secoiridoid glucoside GI 5 in *F. excelsior* (187). A new secoiridoid glycoside, 6′-*O*-β-apiofuranosylsweroside **255**, was identified from the leaves of *Lonicera angustifolia* (188). The novel secoiridoid glycoside 6′-*O*-β-apiofuranosylsweroside **256**, together with the known sweroside **257**, were isolated from the leaves of *L. angustifolia* (189). From Scottish plants, two secoiridoid glycosides, sweroside **257** and swertiamarin **258**, were isolated from the aerial parts of *Centaurium erythraea* (family, Gentianaceae) (190) and the antibacterial, free radical-scavenging activities, and general toxicity of these glycosides were assessed. Both compounds inhibited the growth

of *Bacillus cereus, B. subtilis, Citrobacter freundii*, and *Escherichia coli*. Swertiamarin was also active against *Proteus mirabilis* and *Serratia marcescens*, and sweroside inhibited the growth of *Staphylococcus epidermidis*. Swertiamarin and sweroside exhibited significant general toxicity in a brine shrimp lethality bioassay: The LD_{50} values (dose of a chemical that kills 50% of a sample population) were 8.0 and 34 μg/mL, respectively, whereas that of the positive control podophyllotoxin, a well-known cytotoxic lignan, was 2.79 μg/mL. The chemotaxonomic implications of these compounds in the family Gentianaceae have also been discussed briefly. Gentiopicroside **259**, a secoiridoid glycoside isolated from the aerial parts of *Centaurium erythraea*, was assessed for antibacterial and free radical-scavenging activities (191). The general toxicity of **259** was also detected by a brine shrimp lethality bioassay. Gentiopicroside **259** showed antibacterial activity against *B. cereus*, *B. subtilis*, *Centaurium umbellatum*, *C. freundii*, *Enterococcus faecalis*, *E. coli*, *Klebsiella pneumoniae*, *Lactobacillus plantarum*, *Micrococcus luteus*, *P. mirabilis*, and *Pseudomonas aeruginosa*, and radical-scavenging activity against *Salmonella goldcoast*, *S. marcescens*, *Staphylococcus aureus*, *S. epidermidis*, and *S. hominis*.

A methanolic extract of *Swertia chirata*, which was found to inhibit the catalytic activity of topoisomerase I of *Leishmania donovani*, was subjected to fractionation to yield three secoiridoid glycosides: amarogentin **260**, amaroswerin **261**, and sweroside **257**. Amarogentin **260** was a potent inhibitor of type I DNA topoisomerase from *Leishmania* and exerted its effect by interaction with the enzyme, preventing the formation of binary complexes (192).



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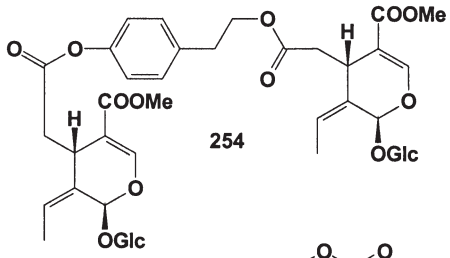

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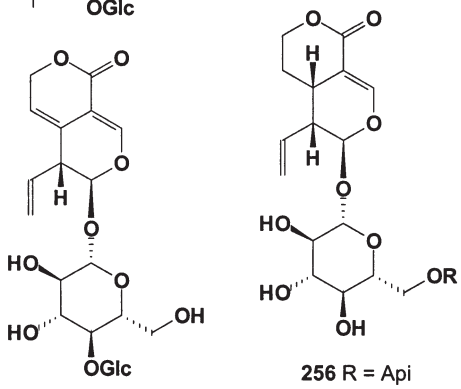
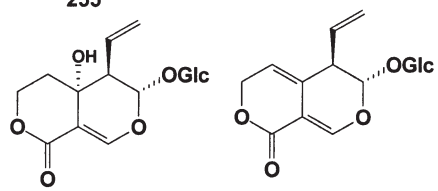


255

256 R = Api
257 R = H



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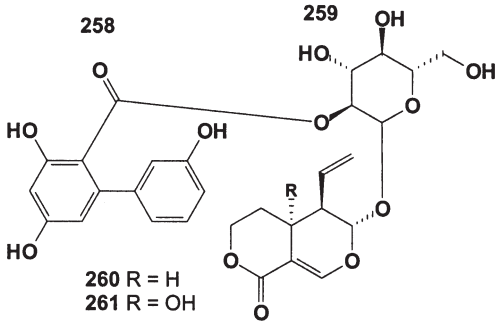
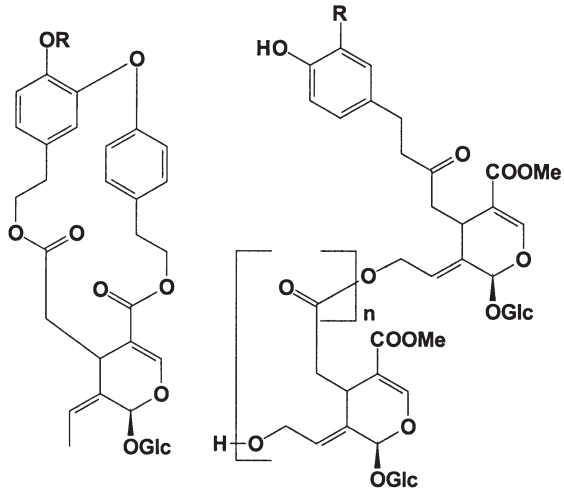
259



260 R = H
261 R = OH

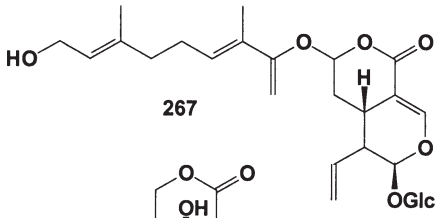
Fraxuhdoside **262** (193) and insularoside **263** (194) were obtained from extracts of *Fraxinus uhdei* and *F. insularis*, respectively. *Fraxinus uhdei* is native to western and southern Mexico, Guatemala, and Honduras and has been planted as an ornamental tree in other countries, including Costa Rica, Puerto Rico, and Hawaii. Its wood is used for baseball bats, paddles, and tool handles. Three new secoiridoid glucosides, jasamplexosides A **264**, B **265**, and C **266**, were isolated from the crude drug Niu Du Teng, the leaves and stems of *Jasminum amplexicaule* (195). In Pakistan, some species belonging to the genus *Jasminum* are usually used as a diuretic, emmenagogue, and

anthelmintic; for the skin, headaches, weak eyes, and scorpion stings; to cure ringworm; for ulcerations or eruptions in the mouth; and for the ears in otorrhea (196). The flowers are used to prepare perfumes. Several *Jasminum* species have been used in cancer treatment. Jasmine flower oil is important in high-grade perfumes and cosmetics, such as creams, oils, soaps, and shampoos. Jasmine is beneficial for the skin, reducing problems such as dry, greasy, irritated, or sensitive skin (it is good for dry, sensitive skin, especially when there is redness or itching). Its flowers are used in jasmine tea and other herbal black and green teas. The roots and leaves of some jasmine species have been used in folk medicine as an anthelmintic that is active against ringworm and tapeworm, and it is also used to treat muscle spasms, sprains, catarrh, coughs, hoarseness, laryngitis, uterine disorders, labor pains, frigidity, depression, and nervous exhaustion. The *Jasminum* spp. have traditionally been considered an aphrodisiac and a calmative. In the Orient the root is used to treat headaches, insomnia, and pain caused by dislocated joints and broken bones (197).

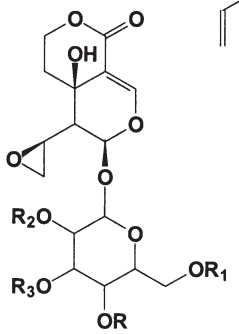


262 R = Glc
263 R = H

264 R = H, n = 1
265 R = H, n = 2
266 R = OH, n = 1



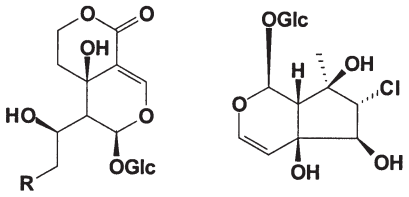
267



268 R = R_1 = R_2 = R_3 = H
269 R = Feruloyl, R_1,R_2,R_3 = H
270 R_1,R_2,R_3 = Ac, R = Fer

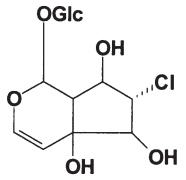
An acylated secoiridoid glucoside of the foliamenthin type, named exaltoside **267**, has been isolated from the aerial parts of the water plant *Villarsia exaltata* (Menyanthaceae) (198). Watt and Breyer-Brandwijk (199) recorded that when prepared as an ointment, the plant has been used in traditional medicine as a remedy for hemorrhoids. Eustomoside **268**, angustioside **269**, gentomoside **270**, eustomorusside **271**, and one chlorine-containing secoiridoid glucoside named eustoside **272** were isolated from species of the Gentianaceae (gentian) family (170). Eustomoside **268** and angustioside **269** were isolated from *Swertia angustifolia* (200), gentomoside **270** was isolated from the seeds of *Styrax perkinsiae* (201), and eustomorusside **271** and eustoside **272** were found in the lisianthus *Eustoma russellianum* (202). Linarioside, a chlorine-containing iridoid glucoside, **273**, was isolated from *Linaria japonica* (Scrophlariaceae) (203,204) and more recently was found in the snapdragon, *Antirrhinum majus* (205). The snapdragon (*A. majus*) has bitter and stimulant properties, and the leaves of this and several allied species have been used on the continental part of Europe in cataplasms for tumors and ulcers. The snapdragon is closely allied to the toadflaxes. Like the toadflax, it was valued in olden times as a preservative against witchcraft. The bitter leaves and flowers are used as antiphlogistics, resolvents, and stimulants. It is effective in the treatment of all kinds of inflammation and is also used on hemorrhoids (206).

Thunbergioside **274**, which has a structure similar to linarioside **273**, was isolated from four species of *Thunbergia* (207), from *Retzia capensis* (208), and from *T. alata* (209) and is also present in plants of the Thunbergioideae, Mendoncioideae, and Nelsonioideae subfamilies (all belonging to Acanthaceae) (210).



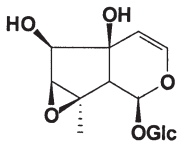
271 R = OH
272 R = Cl

273

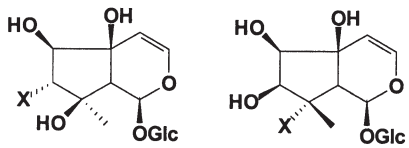


274

Treatment of the iridoid glucoside antirrhinoside **275** with pyridinium chloride in dimethylformamide yielded two possible *trans*-halohydrins, linarioside **273** and isolinarioside **277**. Pyridinium bromide yielded two analogous bromohydrins, **276** and **278** (211). The iridoid glucosides 8-*epi*-muralioside from *Linaria arcusangeli* and 7,8-*epi*-antirrhinoside from *Linaria dalmatica* were both shown to be identical to isolinarioside **277**.



275



273 X = Cl
276 X = Br

277 X = Cl
278 X = Br

SUMMARY

Medicinal plants are widely used nowadays for the preparation of various pharmaceutical formulations or as food additives. Plants produce secondary metabolites, which, among other functions, protect them against microbial and herbivorous attack or UV irradiation. Secondary metabolites also have direct uses for human beings. Hemi- and monoterpenoids, for example, have health-promoting activities as food ingredients, and many iridoid glycosides have pharmacological activities. The chemical features of medicinal plants serve as an integral determinant of their species specificity and pharmacological properties, and enable their wide use in medical practice. The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists and natural-products chemists are combing the earth for phytochemicals and "leads" that could be developed for the treatment of infectious diseases. Traditional healers have long used plants to prevent or cure infectious conditions, and Western medicine is trying to duplicate their successes. Plants are rich in a wide variety of secondary metabolites; hemiterpenoids; acyclic, monocyclic, and bicyclic monoterpenoids; and iridoid glycosides, which have been found to have antimicrobial, anticancer, and other properties.

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Intravenous Application of Omega-3 Fatty Acids in Patients with Active Rheumatoid Arthritis. The ORA-1 Trial. An Open Pilot Study

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ABSTRACT: The objective of this work was to assess the therapeutic efficacy and tolerability of intravenously applied n-3-PUFA in patients with active rheumatoid arthritis (RA). Thirty-four patients with active RA [identified as having a DAS28 (disease activity score including a 28 joint count) > 4.0] were enrolled into this 5-wk open pilot study (one group design). From the time of screening (visit 0, or V0), background therapy had to remain unchanged. Patients received 2 mL/kg (= 0.1–0.2 g fish oil/kg) fish oil emulsion intravenously on 7 consecutive days (Visit 1–Visit 2, or V1–V2) in addition to their background therapy. A decrease of the DAS28 > 0.6 at day 8 (Visit 2) was the primary efficacy measure. Moreover, the DAS28 at day 35 (Visit 3, or V3), the modified Health Assessment Questionnaire, the American College of Rheumatology (ACR) response criteria (V2, V3) and the Short Form-36 (V3) were assessed. Thirty-three patients completed the trial. The mean DAS28 at V1 was 5.45;at V2, 4.51 ($P < .001$ V1–V2) and at V3, 4.73 ($P < .001$ V1–V3; V2–V3, not significantly different). Of the 34 patients, 56% achieved a reduction of the DAS28 > 0.6 at V2 (mean 1.52); 27% > 1.2. At V3, 41% of the patients showed a DAS28 reduction > 0.6 (mean 1.06), and 36% > 1.2. ACR 20 and 50% responses at V2 were seen in 29 and 12% of patients, respectively; at V3, the comparable values were 18 and 9%, respectively. Overall tolerability was excellent. Intravenous application of n-3-PUFA (as an add-on therapy) was considerably well tolerated and led to improvement of the disease activity status in a reasonable number of RA patients. Future trials are warranted to answer whether the intravenous application of n-3-PUFA constitutes a therapeutic option in RA patients.

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Abbreviations: ACR, American College of Rheumatology; BMI, body mass index; CRP, C-reactive protein; DAS28, disease activity score including a 28-joint count; DMARD, disease-modifying antirheumatic drug(s); ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; EULARC, EULAR response criteria; ITT, intent-to-treat; M-HAQ, modified health assessment questionnaire; NS, not significantly different; NSAID. nonsteroidal antirheumatic drug(s); RA. rheumatoid arthritis; RF, IgM-rheumatoid factor; SF-36, Medical Outcomes Study Short Form 36; SJC, swollen joint count; TJC, tender joint count; V0, screening visit; V1, study entry visit; V2, visit after 1 wk; V3, final visit (4 wk after application of PUFA); VAS, visual analog scale; VAS-GH, visual analog scale of patient's general health; VAS pain, visual analog scale of patient's pain; VAS ph, visual analog scale of physician's global assessment;

The habitual lifelong intake of fish and vegetables and dietary measures including the use of olive oil are supposed to exert independent protective effects on the development or severity of rheumatoid arthritis (RA) (1–3). These hypotheses are supported by epidemiological observations from selected geographical regions (4,5).

The n-3 PUFA, EPA and DHA, are highly concentrated in oily fish and fish oils. These n-3 PUFA are structurally and functionally different from n-6 PUFA. In typical cases of human inflammation, the cells involved contain far higher concentrations of the n-6 PUFA arachidonic acid than of n-3 PUFA. Arachidonic acid is the precursor of prostaglandins and leukotrienes, which are known to be highly active mediators of inflammation. Consumption of fish oil causes partial replacement of arachidonic acid in inflammatory cell membranes by EPA (6).

This exchange leads to a decrease of arachidonic acid-derived mediators, resulting in a potentially beneficial anti-inflammatory effect of n-3 PUFA. Moreover, a number of other effects may result from altered eicosanoid production or may be independent of this activity. For example, several animal and human studies demonstrated that the application of fish oil suppresses the synthesis of proinflammatory cytokines and is capable of reducing adhesion molecule expression (6).

In clinical studies, oral supplementation with fish oil reduces signs and symptoms of RA in patients despite ongoing therapy with disease-modifying antirheumatic drug(s) (DMARD) (7). Also, fish oils have protective clinical effects in occlusive cardiovascular disease, for which patients with RA are at increased risk (8,9).

Implementation of the clinical use of anti-inflammatory fish oil doses has been poor, in view of the sometimes only modest beneficial effect of oral supplementation or diets, however evident, and some problems concerning patients' compliance. Secondly, the onset of dietary fish oil supplement efficacy is delayed up to 3–4 mon. To initiate a treatment with such a delayed effect requires belief in the efficacy of the prescriber and often difficult and time-consuming articulation to the patient (10).

Therefore, we intended to assess the efficacy and tolerability of an intravenously administered fish oil emulsion in patients with active RA despite ongoing therapy with disease-modifying agents and corticosteroids in hopes of achieving a rapid onset of efficacy. To our knowledge, this is the first investigation dealing with the intravenous application of highly

unsaturated FA in active RA patients. As this trial constituted the very first attempt, whether this treatment modality was worthwhile for further investigations the one group-design seemed to be one of the appropriate choices to be made in deciding .

Here we report the results of this pilot study.

PATIENTS AND METHODS

All patients gave their written informed consent according to the Declaration of Helsinki. The design of the study has been approved by the local ethics committee.

To be included, patients had to have active RA, according to the American Rheumatism Association criteria (11), defined as a DAS28 (disease activity score including a 28-joint count) (12) higher than 4.0 at the screening visit (visit 0, or V0) despite ongoing treatment, consisting of disease-modifying drugs, corticosteroids up to 10 mg prednisolone/d, and NSAID [nonsteroidal anti-inflammatory drug(s)] on demand. The intake of n-3- and n-6 PUFA and the average joule intake/d in the last week before inclusion were assessed. All patients were told not to change their usual diet until the end of the study (V3), to which they agreed. From V0, no change of the background therapy was allowed during the observation period.

Exclusion criteria were changes in the DMARD regimen, relevant changes (exceeding ±2.5 mg prednisolone equiv/d) in the corticosteroid dosage, oral supplementation of unsaturated FA, and physiotherapy within the last 3 mon. After the screening visit (V0), patients were assessed before the first infusion (V1), after the last infusion at day 8 (V2), and 28 d thereafter (V3).

Patients received 2 mL/kg (= 0.1–0.2 g fish oil/kg) fish oil emulsion (Omegaven®; Fresenius-Kabi, Graz, Austria) intravenously over 4 h on 7 consecutive days in addition to their background therapy. One hundred milliliters of this preparation contains 6.56 g of n-3 PUFA, comprising 2.82 g EPA (20:5n-3), 3.09 g DHA (22:6n-3), 0.20 g a-linolenic acid (18:3n-3), and 0.45 g docosapentaenoic acid (22:5n-3). The change in the DAS28 at V2 constituted the primary efficacy criterion. A reduction of the DAS28 > 0.6 was regarded as a primary response (13). Secondary efficacy criteria comprised the DAS28 change at V3; the American College of Rheumatology (ACR) response rates (14), including the modified Health Assessment Questionnaire (M-HAQ) (15) at V2 and V3; the Medical Outcomes Study Short Form 36 (SF-36) (16) at V3; and the consumption of NSAID at V2 and V3.

Tolerability was assessed by clinical examination; laboratory tests, comprising liver function tests, kidney function tests, complete blood cell count, neutral fat, total cholesterol, HDL- and LDL-cholesterol, fasting blood glucose, and coagulation analysis (Quick-Test, aPTT) and additionally by the reporting of side effects at V2 and V3. The body mass index (BMI) was calculated for each patient at every study phase.

Statistical evaluation. The sample size estimation was based on the pilot data of 16 patients (ΔDAS28 V0→V1: mean = 0.37, SD = 1.0) and on the assumption that ΔDAS28 V1→V3 will be 0.6 lower than ΔDAS28 V0→V1. With a type I error of 5% (two-tailed) and a power of 90%, a sample size of 30 (valid cases for efficacy) was calculated.

Analyses were carried out by applying the McNemar test and Student's *t*-test for dependent variables. For analyzing variables that were not normally distributed, the Wilcoxon-rank test was applied. Data evaluation was purely descriptive without adjustments of the *P*-values. Consequently, significances at $P < .05$ reflect the probability of differences, which can at best be used for generating hypotheses but not for proving them.

RESULTS

Thirty-four patients (27–81 yr; 29 female, 5 male; 25 IgM-rheumatoid factor-positive) were enrolled into this open pilot study [intent-to-treat (ITT) population]. Personal data at baseline are given in Table 1. Thirty-three patients completed the trial; one patient dropped out between V2 and V3 (see Fig. 1). Three more patients were excluded from the per protocol efficacy analysis due to a DAS28 < 4.0 at V1, resulting in a per protocol population of 30 patients.

Two-thirds of the patients were treated as in-patients during the 7-d treatment period, mostly for the distance between the hospital and their residence. No differences could be found between the in-patients and the group of outpatients.

TABLE 1
Personal and Baseline Data of the Patient Population Investigated[a]

	PP-population (n = 30)	ITT-population (V1–V2: n = 34) (V1–V3: n = 33)
Age (yr; median ± 95% CI)	60 [± 4.6]	61 [± 4.2]
Gender (M/F; %)	17/83	15/85
Body weight (kg; median ± 95% CI)	74.5 [± 5.5]	74[± 5.3]
BMI (kg/m^2) (median ± 95% CI)	28.1 [± 4.73]	28.4 [± 4.64]
Disease duration (mon; median ± 95% CI)	105 [± 44.0]	90 [± 45.6]
DAS28 (V0; median ± 95% CI)	5.52 [± 0.27]	5.47 [± 0.26]
M-HAQ (V1; median ± 95% CI)	1.4 [± 0.24]	1.4 [± 0.22]
Morning stiffness (min; median ± 95% CI)	25 [± 26.5]	30 [± 25.3]
Clinically active joint regions (*n*/patient; median ± 95% CI)	2 [± 0.4]	2 [± 0.4]
Arthritis of the hand and finger joints (%)	100	97
Symmetrical arthritis (%)	80	79
Nodules (%)	13	15
RF positivity (%)	76	76
Erosions (%)	76	76

[a]All metric variables are expressed as medians [95% CI in brackets]. PP, per protocol analysis; ITT, intent-to-treat analysis; CI, confidence interval; BMI, body mass index; DAS28, disease activity score including a 28-joint count; M-HAQ, modified health assessment questionnaire; RF, IgM-rheumatoid factor (determined by immunoturbidimetry); V0, screening visit; V1, study entry visit.

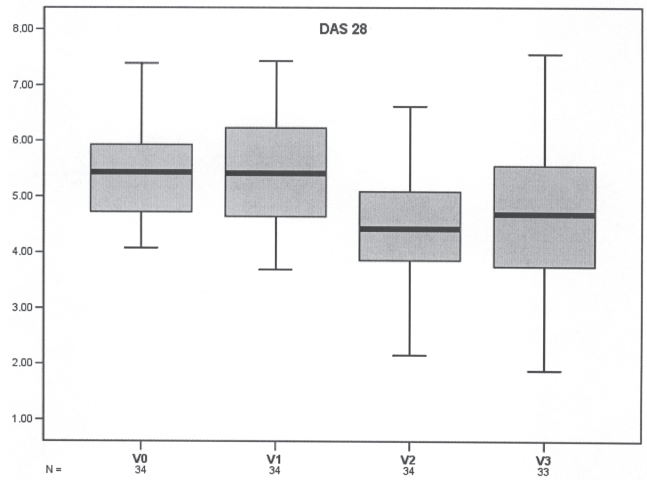

FIG. 1. Box-plots of the DAS28 in the ITT patient population (V1–V2: $n = 34$, V1–V3: $n = 33$; $P < .001$ V1–V2; $P < .001$ V1–V3; not significantly different V2–V3). DAS28, disease activity score including a 28-joint count; ITT, intent-to-treat analysis; VI, study entry visit; V2, second visit, 7 d after V1; V3, third visit, 35 d after V1.

The mean values for the DAS28 in the ITT-population at the screening visit V0 (5.1, 4.08–7.39) and at V1 (5.45; 3.69–7.43) were not significantly different. At V2, immediately after the intervention, the mean DAS28 was 4.51 (2.16–6.62); at V3, 28 d after the intervention, a reincrease to a mean of 4.71 (1.89–7.56) was evident. A highly significant decrease in the DAS28 mean of 0.94 from V1 to V2 ($P < .001$) was apparent that could be maintained to V3 (DAS28 reduction to V1 0.72; $P < .001$), although a tendency to a reincrease [0.20; NS (difference not statistically significant) V2 to V3] indicated a relatively short treatment effect (see Fig. 1).

In fact, 56% of the patients met the limit of a reduction of the DAS28 > 0.6 at V2, whereas at V3 in 41% of the patients this threshold could still be achieved. The 19 patients (17 female) achieving a reduction of the DAS28 > 0.6 at V2 were therefore regarded as primary responders; in 9 patients the DAS28 reduction was >1.2 (27%). At V3, 14 patients showed a DAS28 reduction >0.6, 12 patients >1.2 (36%). The mean DAS28 reduction in the responders amounted to 1.53 between V1 and V2 ($P < .001$) and to 1.06 between V1 and V3, respectively ($P < .001$).

Even in the nonresponders a positive trend toward decreasing disease activity was noted. Indeed, only four patients experienced an increase of the DAS28 (mean 0.19; 0.11–0.35) at V2, whereas at V3 eight patients faced such an increase (mean 0.46; 0.04–0.97) compared with V2. Subgroup analysis of responders and nonresponders failed to demonstrate significant differences between the two groups or reveal predisposing factors for response.

Changes of the single parameters of the DAS28 in the ITT population were as follows. The tender joint count (TJC) mean at V1 was 10 (1–25), decreased to 6 (0–25) at V2 ($P = .015$) and was 7 (0–26) at V3 (NS V1–V3; NS V2–V3).

The mean value for the swollen joint count (SJC) was 7 (0–17) at V1, 5 (0–17) at V2 ($P = .014$), and 5 (0–14) at V3 ($P = .043$ V1–V3; NS V2–V3) see Table 2.

The courses of C-reactive protein (CRP)-levels and the erythrocyte sedimentation rate (ESR) are shown also in Table 2**.** CRP values failed to demonstrate significant differences, whereas ESR showed a significant decrease ($P = .007$ V1–V2, $P = .015$ V1–V3). The patients' global assessment (VAS-GH; visual analog scale of patient's general health) averaged 46 (16–82) at V1, decreased to25 (0–80) at V2 ($P < .001$) and rose to 39 (0–82) at V3 (NS V1–V3; $P < .001$ V2–V3). Physician's global assessment (VAS-ph) and patient's pain assessment (VAS pain) also were found to decrease significantly after the therapeutic intervention (VAS-ph: $P < .001$ V1–V2; $P = .006$ V1–V3; patient's pain: $P < .001$ V1–V2; $P = .019$ V1–V3), see also Table 2**.** Analysis of the per protocol population gave congruent results.

The ACR response rates are given in Table 3. Whereas the M-HAQ did not change significantly throughout the observation period after the administration of the fish oil preparation (Table 2), general health as assessed by the SF-36 improved significantly from V1 to V3 ($P = .015$).

NSAID consumption remained unchanged in 85% of the patients during the observation period; in 9% a reduction and in 6% an increase of the NSAID consumption were evident.

Tolerability. Patients did not report spontaneously any of the unpleasant side effects usually associated with the oral intake

TABLE 2
Disease Activity Parameters and DAS28 at V1, V2, and V3[a]

	V1 mean (± SD)	V2 mean (± SD)	*P*-value V1–V2	V3 mean (± SD)	*P*-value V1–V3
TJC	9.94 (7.19)	6.09 (5.82)	.015	7.33 (7.63)	.303 (NS)
SJC	6.94 (4.22)	4.62 (3.58)	.014	4.82 (3.54)	.043
ESR (1st hour)	40.59 (19.82)	35.00 (18.00)	.007	32.85 (19.12)	.015
CRP (mg/dL)	2.47 (2.32)	2.12 (2.50)	.216 (NS)	2.18 (2.41)	.683 (NS)
VAS GH (0–100)	45.59 (17.72)	25.18 (21.53)	<.001	39.39 (23.23)	.094 (NS)
VAS ph (0–100)	46.35 (22.38)	23.24 (19.89)	<.001	34.03 (23.11)	.006
VAS pain (0–100)	49.24 (20.08)	29.74 (24.19)	<.001	39.27 (22.38)	.019
M-HAQ (0–3)	1.36 (0.68)	1.24 (0.76)	.09 (NS)	1.30 (0.71)	.531 (NS)
DAS28	5.45 (1.05)	4.51 (1.05)	<.001	4.71 (1.33)	<.001

[a]Values are expressed as means ± SD. TJC, tender joint count out of a 28-joint count; SJC, swollen joint count out of a 28-joint count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; VAS-GH, visual analog scale of patient's general health; VAS ph, visual analog scale of physician's global assessment; VAS pain, visual analog scale of patient's pain; NS, not significantly different; for other abbreviations see Table 1.

TABLE 3
ACR Response Rates in the Entire Patient Cohort[a]

	PP-population (n = 30)	ITT-population (V1–V2: n = 34, V1–V3: n = 33)
ACR 20 (V1–V2) (%)	30	29
ACR 50 (V1–V2) (%)	10	12
ACR 70 (V1–V2) (%)	7	6
ACR 20 (V1–V3) (%)	17	18
ACR 50 (V1–V3) (%)	7	9
ACR 70 (V1–V3) (%)	3	3

[a]ACR, American College of Rheumatology; for other abbreviations see Table 1.

of unsaturated FA such as fishy halitosis or fishy body odor (17). Among the measured blood parameters, no significant differences between V1 and V3 could be observed. Only LDL-cholesterol changed significantly; an initial increase was followed by a final decrease at V3, matching well the experimental experience of the antihypercholesterolemic action of omega-6 FA (18). The BMI did not change significantly throughout the trial.

Overall tolerability, including local reactions at the infusion site, was excellent. One patient dropped out because of a severe flare-up of the disease, another severe adverse event was caused by an acute lumbar disc herniation. Three upper respiratory tract infections and one case of local phlebitis were also observed. No adverse effect was assessed as being putatively drug-related.

DISCUSSION

To our knowledge, this pilot trial constitutes the first investigation dealing with the intravenous administration of n-3 PUFA, namely, a fish-oil emulsion containing high amounts of EPA and DHA, in patients with active RA. The preparation, Omegaven, is licensed for parenteral nutrition therapy. In contrast to the published trials applying capsules or diets (19,20), no problems with patients' compliance, factors possibly influencing the absorption of the compounds, or gastrointestinal intolerance were to be expected in case of intravenous administration. Moreover, the doses applied during this trial were apparently higher than those that were used during preceding investigations. A major difference between oral and intravenous therapy is cost, with Omegaven treatment likely to be far more expensive on the basis of the actual price for the preparation. However, a formal cost-effectiveness calculation needs to be performed on the assumption of reduced costs for Omegaven, if it is to be routinely administered in a larger patient population.

As a first result we found a mean reduction of the DAS28 at V2 and V3 in the entire patient population exceeding 0.6, which represents the threshold of the European League Against Rheumatism response criteria (EULARC) (13) for a moderate therapeutic response in patients at medium disease activity. Moreover, a primary response in about 60% of the patients with a mean DAS28 reduction of about 1.5, which is considerably higher than a moderate response according to the EULARC (13), was observed.

This response rate, as measured by the DAS28, which strongly reflects patient's satisfaction (21) and was just recently proposed for use in clinical trials (22), gives evidence for a mixed effect (drug + placebo) rather than for a simple placebo effect. Response as expressed by the ACR criteria was considerably lower, with an ACR 20% response in about 30% of the patients; although relatively low in this short-term observation, this response exceeds the placebo response rates in blinded RA trials (23,24).

Interestingly, parameters such as the TJC, the SJC, the VAS pain, and the VAS-GH declined significantly, whereas the acute phase reactants, particularly CRP levels, did not change appreciably (Table 2). One explanation could be that the relatively short-term application of n-3 PUFA resulted in insufficient substitution of arachidonic acid within the cell membranes. This hypothesis is supported by a common feature of the patients' nutrition, the consumption of a relatively high concentration of n-6 PUFA, prior to the investigation (25).

The other explanation could be a placebo effect, which is inherent in open, noncontrolled trials. Considering the nature of the intervention, namely, intravenous infusion, the placebo effect could be higher than usually seen with oral interventions. Thus, the results of the study, while encouraging, have to be interpreted cautiously with regard to the placebo effect. However, a time-dependent reincrease of the disease activity parameters between V2 and V3 constituted a greater effect than is generally found with a placebo.

Compared with a recently published trial dealing with the therapeutic efficacy of Mediterranean diet vs. ordinary Western diet, the mean DAS28 reduction in all patients in our investigation was almost twice as high and significant according to the EULARC (0.94 vs. 0.56) when compared with the DAS28 reduction in the Mediterranean diet group (20). Moreover, the decrease was achieved far more rapidly, namely, after 1 wk, whereas in the aforementioned trial the DAS28 decreased even after week 6. In both trials a significant amelioration of the patients' general health, as expressed by the SF-36, was seen, whereas in our investigation the M-HAQ did not change significantly. It is doubtful, however, that the M-HAQ constitutes a tool for monitoring disease activity of RA (26).

Two previous blinded trials compared the efficacy of oral fish-oil supplementation at dosages of 40 mg/kg/d for 15 wk and 130 mg/kg/d for 30 wk (27,28). As the DAS28 was not included in those trials a comparison can only be drawn on the basis of the single activity parameters. In both investigations the TJC and patient's global assessment decreased significantly throughout the observation period, yet much slower as in the case of intravenous administration.

Thus, the primary question—whether intravenous administration of n-3 PUFA leads to a significantly earlier treatment effect—could be answered positively. As in preceding trials with oral preparations or diets, a positive treatment effect could be achieved, but it was far more pronounced as measured by the EULARC and occurred far earlier (20,27,28). Moreover, even in the nonresponders a positive trend to a decrease of RA activity was observed. Regardless of the consequences on RA

activity, an additional advantage of n-3 PUFA substitution (10) for RA patients is that it reduces the risk of cardiovascular disease (29), to which they are more susceptible. Given the excellent tolerability of the intravenously administered fish oil emulsion, as shown here, it seems worthwhile to investigate further the therapeutic potential of such preparations in RA patients. As the treatment effect of the intravenous application was relatively short, but occurred rapidly, the administration of repeated treatment cycles should be carefully investigated including blinded study designs. Another possibility to be evaluated would be an initial intravenously applied loading dose of n-3 PUFA followed by sustained long-term oral administration. However, the efficacy of all dietary or orally administrable therapeutic interventions depends far more on the patient's compliance and nutritional habits than intravenously applicable therapeutic regimes.

In summary this pilot trial of intravenous administration of n-3 PUFA revealed moderate short-term efficacy with rapid onset and excellent tolerability. Besides the great progress in the treatment of RA during the last decade (30,31), the therapeutic administration of n-3 PUFA may constitute another possibly effective and presumably safe option for treating RA, for treating other inflammatory rheumatic diseases, and perhaps for reducing patients' cardiovascular risk (17,32).

COMPETING INTERESTS

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n-3 Polyunsaturated Fatty Acids Endogenously Synthesized in Fat-1 Mice Are Enriched in the Mammary Gland

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ABSTRACT: In this study, we determined the phospholipid FA composition in the mammary gland of the transgenic Fat-1 mouse. This is the first animal model developed that can endogenously synthesize n-3 PUFA. The synthesis of n-3 PUFA is achieved through the expression of the *fat-1* transgene encoding for an n-3 desaturase from *Caenorhabditis elegans*, which utilizes n-6 PUFA as substrate. Wild-type and Fat-1 female mice were terminated at 7 wk of age and the fifth mammary gland was removed. Lipids were extracted and phospholipids were separated by TLC and converted to FAME for analysis by GC. There was no significant change in total saturated, monounsaturated, and PUFA composition. However, there was a significant increase in total n-3 PUFA and a corresponding decrease in n-6 PUFA. The major n-3 PUFA that were enriched included 20:5n-3 and 22:6n-3. The n-6 PUFA that were reduced included 20:4n-6, 22:4n-6, and 22:5n-6. Overall, these findings demonstrate that female Fat-1 mice have elevated levels of n-3 PUFA in the mammary gland. Moreover, the n-3 desaturase products are the same n-3 PUFA found in fish oil, which have been shown to have chemoprotective properties against breast cancer. Therefore, this newly developed mouse model may be highly useful for investigating molecular and cellular mechanisms by which n-3 PUFA prevents and inhibits breast cancer growth.

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Studies have shown that n-3 PUFA may prevent or inhibit the growth of breast cancer cells. Results from epidemiological studies have been equivocal (1–4). However, there is stronger evidence from animal and cell culture studies demonstrating that n-3 PUFA can delay the onset of tumor development, reduce the tumor burden, and induce tumor cell death (5–10). Currently, our understanding of mechanisms of action is poorly understood. Many mechanisms have been proposed, including the modulation of eicosanoid synthesis, cell cycle regulatory proteins, gene expression, and alterations in membrane structure and function (11–16). However, relevant transgenic animal models have been lacking that may have important utility for gaining insight into mechanisms of action.

Kang *et al.* (17) recently described the development of the transgenic Fat-1 mouse. This mouse contains the *fat-1* gene from *Caenorhabditis elegans*, which encodes for an n-3 desaturase that can synthesize n-3 PUFA from 18–22 carbon n-6 PUFA. Levels of n-3 PUFA are reportedly increased in many major tissues, including the muscle, liver, heart, erthyrocytes, brain, kidney, and spleen (17). However, the FA composition of the female mammary gland was not reported. In this study we report the detailed FA composition of the major phospholipid fractions in the mammary gland of female Fat-1 transgenic mice.

MATERIALS AND METHODS

Animals and diet. All mice were housed in a temperature- and humidity-controlled animal facility with a 12-h light/dark cycle. Male Fat-1 mice were obtained from Dr. Jing Kang (Harvard Medical School). Male Fat-1 mice were mated with C57Bl/6 female mice (Charles River Laboratories, Wilmington, MA). Fat-1 mice convert n-6 PUFA to longer-chain n-3 PUFA; thus, these mice may potentially become n-6 PUFA deficient. To prevent deficiency, all mice were fed a modified AIN-93G diet containing 10% safflower oil high in 18:2n-6 (Product #D04092701; Research Diets, New Brunswick, NJ). At 7 wk of age, female wild-type ($n = 3$) and Fat-1 ($n = 3$) mice were terminated by CO_2 asphixiation and the fifth mammary gland was removed for FA analysis. All procedures were done in accordance with the animal experimentation guidelines of the University of Toronto.

Analysis of phospholipid FA. Total lipids were extracted from the mammary gland by the method of Folch *et al.* (18). TLC plates (Cat. #10011; Analtech, Newark, DE) were activated by heating at 100°C for 1 h. Phospholipid fractions were separated by developing with chloroform/MeOH/2-propanol/KCl (0.25% wt/vol)/triethylamine (30:9:25:6:18, by vol) (19). Bands corresponding to PC, PS, PI, and PE were visualized under UV light after lightly spraying with 8-anilino-1-naphthalene sulfonic acid (0.1% wt/vol). Bands were scraped and converted to FAME with 14% boron trifluoride/methanol at 100°C for 1 h. FAME were quantified on an Agilent 6890N gas chromatograph equipped with an FID and separated on an Agilent J&W fused-silica capillary column (DB-23; 30 m, 0.25 μm film thickness, 0.25 mm i.d.; Agilent, Palo Alto, CA). Samples were injected in splitless mode. The injector and detector ports were set at 250°C. FAME were eluted using a temperature program set initially at 50°C and held for 2 min, increased at 20°C/min and held at 170°C for 1 min, increased at 3°C/min, and held at 212°C for 10 min to complete the run. The carrier gas was helium, set to a 0.7 mL/min constant flow rate.

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Statistical analyses. Differences in the level of FA between wild-type and Fat-1 mice were compared using Student's *t*-test. Results are expressed as means ± SD. $P < 0.05$ was considered significant.

RESULTS

Animal and tissue weights. There was no significant difference in animal body weights between Fat-1 and wild-type mice (data not shown). However, there was a significant ($P < 0.01$) 28% reduction in the weight of Fat-1 mammary glands relative to those of wild-type mice.

Composition of phospholipid FA in the mammary gland. Levels of total saturated, monounsaturated, and polyunsaturated FA were not different between Fat-1 and wild-type mice. However, there were marked differences in the level of specific n-3 and n-6 PUFA. In particular, levels of EPA (20:5n-3) and DHA (22:6n-3) in Fat-1 mice were significantly increased in all phospholipid fractions relative to those of wild-type mice (Tables 1–4). Concomitantly, there was a significant decrease in total n-6 PUFA in PE, and no significant difference was observed in PC, PS, and PE in Fat-1 mice relative to wild-type mice. Reductions were mainly observed in 20:4n-6, 22:4n-6, and 22:5n-6. Consequently, the n-6 to n-3 ratio was markedly reduced in all phospholipid fractions in Fat-1 mice (Tables 1–4).

DISCUSSION

Two previous *in vitro* studies have demonstrated that adenoviral transfer of the *fat-1* gene into breast cancer and mouse mammary cells can increase n-3 PUFA content (20,21). Although these cell lines have utility for studying mechanisms, an animal model would be of greater utility for integrative studies. We have demonstrated that transgenic Fat-1 mice, which contain a heritable n-3 desaturase from *C. elegans*, have higher levels of n-3 PUFA in phospholipids of the mammary gland relative to those of wild-type mice. The Fat-1 mouse represents a

TABLE 1
Mouse Mammary Gland PC FA Composition (% of total FA)[a]

FA	Wild-type	Fat-1
12:0	0.2 ± 0.04	0.5 ± 0.5
14:0	0.5 ± 0.1	0.3 ± 0.3
15:0	0.1 ± 0.02	0.1 ± 0.1
16:0	21.3 ± 3.0	21.6 ± 2.2
16:1n-7	1.1 ± 0.1	1.0 ± 0.2
18:0	21.5 ± 0.8	21.0 ± 0.8
18:1n-9	8.2 ± 0.4	9.3 ± 0.9
18:1n-7	1.4 ± 0.3	1.3 ± 0.1
18:2n-6	31.9 ± 2.3	34.4 ± 4.1
18:3n-6	0.1 ± 0.03	0.1 ± 0.1
18:3n-3	0.02 ± 0.02	0.2 ± 0.1*
20:0	0.1 ± 0.01	0.1 ± 0.1
20:1n-9	0.2 ± 0.03	0.2 ± 0.1
20:2n-6	0.7 ± 0.1	0.7 ± 0.2
20:3n-6	0.9 ± 0.1	0.7 ± 0.1
20:4n-6	9.3 ± 0.9	5.8 ± 1.5*
20:3n-3	0.2 ± 0.3	0.04 ± 0.03
20:5n-3	0.1 ± 0.2	0.8 ± 0.2*
22:0	0.1 ± 0.1	0 ± 0
22:1n-9	0.1 ± 0.02	0.03 ± 0.02
22:2n-6	0.02 ± 0.02	0.02 ± 0.02
22:4n-6	0.6 ± 0.03	0.3 ± 0.3
22:5n-6	0.9 ± 0.2	0.1 ± 0.1*
22:3n-3	0 ± 0	0 ± 0
24:0	0.1 ± 0.1	0.1 ± 0.1
22:5n-3	0.04 ± 0.1	0.4 ± 0.1*
22:6n-3	0.2 ± 0.1	1.0 ± 0.2*
24:1	0.1 ± 0.1	0 ± 0
Total saturates	44.0 ± 2.9	43.6 ± 1.9
Total monosaturates	11.1 ± 0.6	11.8 ± 1.0
Total polyunsaturates	45.0 ± 3.1	44.5 ± 1.5
Sum of n-6 FA	44.4 ± 3.2	42.2 ± 1.9
Sum of n-3 FA	0.5 ± 0.3	2.3 ± 0.5*
n-6/n-3 ratio	111.3 ± 83.2	18.5 ± 4.2*,[a]

[a]Results are given as mean ± SD. *Significantly different ($P < 0.05$) between wild-type and Fat-1 mice. [a]The n-6 to n-3 ratio is presented but is only significantly different when expressed as the n-3 to n-6 ratio.

TABLE 2
Mouse Mammary Gland PE FA Composition (% of total FA)[a]

FA	Wild-type	Fat-1
12:0	0.4 ± 0.2	0.8 ± 0.8
14:0	0.7 ± 0.5	0.6 ± 0.3
15:0	0.1 ± 0.03	0.1 ± 0.03
16:0	11.6 ± 4.3	8.8 ± 0.5
16:1n-7	2.2 ± 1.6	1.3 ± 0.6
18:0	14.4 ± 7.9	17.0 ± 2.9
18:1n-9	15.5 ± 3.7	15.1 ± 1.0
18:1n-7	1.1 ± 0.2	1.0 ± 0.2
18:2n-6	29.9 ± 13.0	26.7 ± 4.3
18:3n-6	0.1 ± 0.03	0.1 ± 0.1
18:3n-3	0.1 ± 0.1	0.2 ± 0.1
20:0	0.3 ± 0.2	0.4 ± 0.1
20:1n-9	0.4 ± 0.1	0.5 ± 0.1
20:2n-6	0.5 ± 0.1	0.6 ± 0.2
20:3n-6	0.7 ± 0.3	0.7 ± 0.2
20:4n-6	14.8 ± 10.1	13.9 ± 2.5
20:3n-3	0.1 ± 0.1	0.1 ± 0.1
20:5n-3	0.1 ± 0.2	1.7 ± 0.2*
22:0	0.1 ± 0.1	0 ± 0
22:1n-9	0.1 ± 0.04	0.1 ± 0.1
22:2n-6	0.01 ± 0.01	0.02 ± 0.02
22:4n-6	2.1 ± 1.4	1.7 ± 0.5
22:5n-6	3.6 ± 2.6	1.1 ± 0.4
22:3n-3	0 ± 0	0 ± 0
24:0	0.1 ± 0.04	0.1 ± 0.1
22:5n-3	0.2 ± 0.3	1.8 ± 0.3*
22:6n-3	0.8 ± 0.8	5.5 ± 0.4*
24:1	0 ± 0	0 ± 0
Total saturates	27.8 ± 3.2	27.9 ± 1.8
Total monosaturates	19.3 ± 5.4	18.0 ± 1.5
Total polyunsaturates	52.9 ± 2.5	54.0 ± 1.2
Sum of n-6 FA	51.6 ± 2.7	44.7 ± 1.2*
Sum of n-3 FA	1.3 ± 1.4	9.3 ± 0.9*
n-6/n-3 ratio	36.3 ± 26.5	4.9 ± 0.6*,[a]

[a]Results are given as mean ± SD. *Significantly different ($P < 0.05$) between wild-type and Fat-1 mice. [a]The n-6 to n-3 ratio is presented but is only significantly different when expressed as the n-3 to n-6 ratio.

TABLE 3
Mouse Mammary Gland PI FA Composition (% of total FA)[a]

FA	Wild-type	Fat-1
12:0	1.0 ± 0.1	1.6 ± 1.3
14:0	0.7 ± 0.2	0.9 ± 0.7
15:0	0.1 ± 0.1	0.1 ± 0.1
16:0	8.3 ± 0.4	8.5 ± 1.4
16:1n-7	1.0 ± 0.4	0.8 ± 0.5
18:0	29.0 ± 7.9	28.7 ± 5.8
18:1n-9	10.7 ± 1.8	12.3 ± 3.8
18:1n-7	0.9 ± 0.3	0.7 ± 0.1
18:2n-6	12.2 ± 8.6	16.4 ± 6.5
18:3n-6	0.2 ± 0.4	0.1 ± 0.1
18:3n-3	0.1 ± 0.1	0.1 ± 0.1
20:0	0.6 ± 0.5	0.2 ± 0.2
20:1n-9	0.3 ± 0.1	0.2 ± 0.3
20:2n-6	0.4 ± 0.1	0.6 ± 0.2
20:3n-6	0.9 ± 0.3	1.0 ± 0.2
20:4n-6	28.0 ± 5.3	23.9 ± 6.7
20:3n-3	0.1 ± 0.03	0.05 ± 0.1
20:5n-3	0 ± 0	0.8 ± 0.3*
22:0	0.3 ± 0.3	0 ± 0
22:1n-9	0.3 ± 0.3	0.1 ± 0.1
22:2n-6	0.1 ± 0.2	0.01 ± 0.02
22:4n-6	1.8 ± 0.8	0.5 ± 0.5
22:5n-6	2.1 ± 2.5	0.3 ± 0.3
22:3n-3	0 ± 0	0 ± 0
24:0	0.1 ± 0.1	0.1 ± 0.1
22:5n-3	0.1 ± 0.1	0.5 ± 0.4
22:6n-3	0.4 ± 0.1	1.7 ± 0.4*
24:1	0.1 ± 0.2	0.1 ± 0.1
Total saturates	40.3 ± 8.3	40.1 ± 4.5
Total monosaturates	13.2 ± 2.4	14.1 ± 4.3
Total polyunsaturates	46.5 ± 6.0	45.8 ± 3.7
Sum of n-6 FA	45.8 ± 5.9	42.7 ± 1.9
Sum of n-3 FA	0.7 ± 0.1	3.0 ± 0.5*
n-6/n-3 ratio	64.7 ± 4.8	14.3 ± 1.2*

[a]Results are given as mean ± SD. *Significantly different ($P < 0.05$) between wild-type and Fat-1 mice.

TABLE 4
Mouse Mammary Gland PS FA Composition (% of total FA)[a]

FA	Wild-type	Fat-1
12:0	0.9 ± 0.5	1.7 ± 1.3
14:0	0.7 ± 0.4	1.3 ± 1.4
15:0	1.0 ± 1.2	0.1 ± 0.1
16:0	6.4 ± 0.9	7.7 ± 2.0
16:1n-7	0.4 ± 0.1	0.9 ± 0.4
18:0	34.4 ± 1.2	28.6 ± 8.6
18:1n-9	10.9 ± 2.8	14.8 ± 3.1
18:1n-7	0.8 ± 0.01	0.8 ± 0.1
18:2n-6	10.2 ± 2.2	21.3 ± 5.4*
18:3n-6	0.02 ± 0.03	0.03 ± 0.1
18:3n-3	0.2 ± 0.3	0.1 ± 0.1
20:0	0.3 ± 0.04	0.2 ± 0.2
20:1n-9	0.3 ± 0.1	0.3 ± 0.3
20:2n-6	0.4 ± 0.03	0.5 ± 0.2
20:3n-6	1.3 ± 0.4	1.3 ± 0.2
20:4n-6	20.2 ± 9.3	7.4 ± 1.6
20:3n-3	0.1 ± 0.1	0 ± 0
20:5n-3	0 ± 0	0.8 ± 0.4*
22:0	0.5 ± 0.1	0 ± 0*
22:1n-9	0.2 ± 0.1	0.2 ± 0.1
22:2n-6	0.01 ± 0.02	0.02 ± 0.03
22:4n-6	2.8 ± 0.8	1.6 ± 0.5
22:5n-6	6.0 ± 2.1	1.4 ± 0.4*
22:3n-3	0 ± 0	0 ± 0
24:0	0.4 ± 0.1	0.4 ± 0.3
22:5n-3	0.2 ± 0.3	1.5 ± 0.3*
22:6n-3	1.2 ± 0.8	7.0 ± 1.3*
24:1	0 ± 0	0 ± 0
Total saturates	44.6 ± 1.1	39.9 ± 5.1
Total monosaturates	12.6 ± 3.0	17.1 ± 3.5
Total polyunsaturates	42.7 ± 3.7	43.0 ± 1.6
Sum of n-6 FA	41.0 ± 4.3	33.6 ± 3.2
Sum of n-3 FA	1.7 ± 0.9	9.4 ± 1.8*
n-6/n-3 ratio	29.1 ± 13.2	3.7 ± 1.0*

[a]Results are given as mean ± SD. *Significantly different ($P < 0.05$) between wild-type and Fat-1 mice.

significant advance in the development of a more sophisticated research model to investigate mechanisms of action in an intact animal system.

The consumption of fish rich in n-3 PUFA is recommended for its health benefits to protect against heart disease, diabetes, and potentially cancer (22). The major n-3 PUFA enriched in phospholipids were EPA and DHA. These are also the major n-3 PUFA found in fish oil, and they have been shown to be incorporated into human breast tissue and rodent mammary glands (23–25) and to experimentally inhibit breast cancer cell growth (5–10). Most important, levels of total n-3 PUFA in Fat-1 mice, which ranged between ~2 and 9%, were similar in magnitude to levels found in total lipid extracts of human breast adipose tissue, which comprise 1% of total lipids (23). The levels of EPA and DHA reported herein for PC, PE, and PI are remarkably similar to levels found in the same phospholipid classes from R3230AC mammary tumor cells that were implanted in rats fed a fish oil diet (2.5% w/w of diet) for 2–3 wk (25). Collectively, these observations suggest that the Fat-1 mouse has utility as a model to study the role of n-3 PUFA in breast cancer because (i) EPA and DHA, the relevant FA in the human diet that confer protection, are enriched in Fat-1 mammary phospholipids, and (ii) the level of incorporation is comparable to levels found in humans and in conventional feeding studies.

In contrast to n-3 PUFA, there is experimental evidence suggesting that n-6 PUFA promotes the development of mammary carcinogenesis, tumor growth, and metastasis (10,26,27). Therefore, another consideration is the apparently opposing effects of n-3 and n-6 PUFA, which suggests that the relative ratio between n-6 and n-3 PUFA is an important determinant in the overall health benefits of consuming n-3 PUFA (4,28). The n-6 to n-3 ratio consumed in the present Western diet is between 10:1 and 20:1; however, our ancestors had a diet closer to 1:1 (29). These observations suggest that a lower n-6 to n-3 ratio may be optimal for one's health. Analyses of breast adipose tissue from breast cancer patients and controls have shown that a high n-6 to n-3 ratio was correlated with increased risk, whereas a lower ratio reduced the relative risk of breast cancer (23,24). We observed a marked reduction in the n-6 to n-3 ratio, ranging between 111 and 29 and between 19 and 4 in wild-type and Fat-1 mice, respectively. The importance of the n-6 to n-3 ratio is another characteristic that can be studied in this animal model.

The significant ($P < 0.05$) 28% reduction in the weight of the Fat-1 mammary gland suggests that increased levels of n-3 PUFA had a profound impact on the growth and development of the mammary gland. Currently, the implications of this observation are unknown, and ongoing investigations are focused on understanding the effect of n-3 PUFA on mammary gland development.

Modification of membrane FA composition is one of the mechanisms by which n-3 PUFA may protect or inhibit tumor cell growth. Consequently, this influences the synthesis of eicosanoids from arachidonic acid (20:4n-6), which may enhance tumor growth and metastasis (8,27). Changes in eicosanoid synthesis are likely, given that there was a trend toward higher levels of linoleic acid and reduced levels of arachidonic acid in Fat-1 mice, suggesting that elongation and desaturation were inhibited by the presence of n-3 PUFA. This pattern was also present in bone and muscle tissue (data not shown). Although yet to be investigated in breast cancer, changes in membrane FA composition may also potentially influence caveolae-mediated signaling (15,16) and Akt signaling mediated by DHA incorporation into PS (30). These novel mechanisms may be investigated using cell culture systems or rodent feeding studies. However, the transgenic Fat-1 mouse model has unique properties that may be considered advantageous over conventional rodent model systems.

Cell culture systems are certainly powerful systems for studying mechanisms but may not be physiologically relevant, e.g., for a given dose of treatment. Therefore, it is also necessary to conduct parallel dietary feeding studies using intact animal model systems. However, dietary fat studies are confounded by the need to formulate isocaloric diets with respect to fat content. For example, to create a control diet without n-3 PUFA and an experimental diet with high n-3 PUFA, the addition of n-3 PUFA would require a compensatory reduction in another type of fat, such as n-6 PUFA. Thus, it could be argued that any change observed is due to either an increase in n-3 PUFA or a reduction in n-6 PUFA. In addition, to formulate diets with different n-6 to n-3 ratios requires the blending of several oil sources; thus, the fat composition between control and experimental diets is difficult to control. The Fat-1 mouse model is not subject to these potential confounders, given that Fat-1 mice can endogenously synthesize n-3 PUFA; thus, only one diet need be provided to both wild-type and Fat-1 mice. These considerations make the Fat-1 mouse an attractive model system that can provide more conclusive evidence demonstrating the beneficial effects of n-3 PUFA.

Overall, this study demonstrated that the Fat-1 mouse has high levels of n-3 PUFA in mammary gland phospholipids and a low n-6 to n-3 ratio relative to that of wild-type mice. In addition, the strengths of this mouse model suggest that it will have great utility in future investigations for studying molecular and cellular mechanisms by which n-3 PUFA protects against breast cancer.

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Conjugated Linoleic Acid Isomers Reduce Blood Cholesterol Levels but Not Aortic Cholesterol Accumulation in Hypercholesterolemic Hamsters

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ABSTRACT: The aim of the present study was to characterize plasma lipids and lipoprotein cholesterol and glucose concentrations in hamsters fed either *cis*-9,*trans*-11 CLA (9*c*,11*t* CLA); *trans*-10,*cis*-12 CLA (10*t*,12*c* CLA); or linoleic acid (LA) on the accumulation of aortic cholesterol in hypercholesterolemic hamsters. One hundred male F_1B strain Syrian Golden Hamsters (*Mesocricetus auratus*) (BioBreeders Inc., Watertown, MA) approximately 9 wk of age were housed in individual stainless steel hanging cages at room temperature with a 12-h light/dark cycle. Hamsters were given food and water *ad libitum*. Following a 1-wk period of acclimation, the hamsters were fed a chow-based (nonpurified) hypercholesterolemic diet (HCD) containing 10% coconut oil (92% saturated fat) and 0.1% cholesterol for 2 wk. After an overnight fast, the hamsters were bled and plasma cholesterol concentrations were measured. The hamsters were then divided into 4 groups of 25 based on similar mean plasma VLDL and LDL cholesterol (nonHDL-C) concentrations. Group 1 remained on the HCD (control). Group 2 was fed the HCD plus 0.5% 9*c*,11*t* CLA isomer. Group 3 was fed the HCD plus 0.5% 10*t*,12*c* CLA isomer. Group 4 was fed the HCD plus 0.5% LA. Compared with the control, both CLA isomers and LA had significantly lower plasma total cholesterol and HDL cholesterol concentrations ($P < 0.001$) after 12 but not 8 wk of treatment and were not significantly different from each other. Also, both CLA isomers had significantly lower plasma nonHDL-C concentrations ($P < 0.01$) compared with the control after 12 but not 8 wk of treatment and were not significantly different from each other or the LA-fed hamsters. Plasma TG concentrations were significantly higher ($P < 0.004$) with the 10*t*,12*c* CLA isomer compared with the other treatments at 8 but not at 12 wk of treatment. Plasma TG concentrations were also significantly lower ($P < 0.03$) with the 9*c*,11*t* CLA isomer compared with the control at 12 wk of treatment. Also, the 10*t*,12*c* CLA isomer and LA had significantly higher plasma glucose concentrations compared with the control and 9*c*,11*t* CLA isomer ($P < 0.008$) at 12 wk of treatment, whereas at 8 wk, only the LA treatment had significantly higher plasma glucose concentrations ($P < 0.001$) compared with the 9*c*,11*t* CLA isomer. Although liver weights were significantly higher in 10*t*,12*c* CLA isomer-fed hamsters, liver total cholesterol, free cholesterol, cholesterol ester, and TG concentrations were significantly lower in these hamsters compared with hamsters fed the control, 9*c*,11*t* CLA isomer, and LA diets ($P < 0.05$). The 9*c*,11*t* CLA isomer and LA diets tended to reduce cholesterol accumulation in the aortic arch, whereas the 10*t*,12*c* CLA isomer diet tended to raise cholesterol accumulation compared with the control diet; however, neither was significant. In summary, no differences were observed between the CLA isomers for changes in plasma lipids or lipoprotein cholesterol concentrations. However, the 9*c*,11*t* CLA isomer did appear to lower plasma TG and glucose concentrations compared with the 10*t*,12*c* CLA isomer. Such differences may increase the risk of insulin resistance and type 2 diabetes in humans when the 10*t*,12*c* CLA isomer is fed separately.

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Animal studies in our laboratory as well as in others have shown that feeding a predominantly linoleic acid (LA)-containing diet such as corn oil or safflower oil results in dramatic decreases in blood LDL cholesterol (LDL-C) concentrations, which are associated with reductions in atherosclerosis (1,2). However, in these experiments blood LDL-C is reduced so dramatically that the nature of the LDL is unimportant. In contrast, in human studies, the magnitude of the blood LDL-C reduction with LA-rich diets is usually on the order of 5–10%; thus, these LDL particles enriched in LA are more susceptible to oxidation (3) and are presumably more atherogenic (4). Support for the atherogenicity of LA-enriched LDL comes from studies recently completed in our laboratory in which hypercholesterolemic animals fed a diet enriched in sunflower oil (LA-containing) and cholesterol had dramatically more aortic atherosclerosis than animals fed TriSun® oil (oleic-containing; SVO Enterprises, Eastlake, OH) plus cholesterol despite similar, albeit elevated, LDL-C levels (5). Moreover, the increase in aortic atherosclerosis with sunflower oil feeding was highly associated with greater LDL oxidative susceptibility and accumulation of aortic oxidized LDL (5).

These observations beg the question of whether CLA would be more effective than LA in terms of reducing the progression of the aortic lesion. Although several studies have confirmed the anticancer properties of CLA, only a few published reports support its antiatherosclerotic effects, and only one has shown the influence of the purified 9-*cis*,11-*trans* (9*c*,11*t*) CLA iso-

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Abbreviations: 9*c*,11*t* CLA, *cis*-9,*trans*-11 CLA; 10*t*,12*c* CLA, *trans*-10,*cis*-12 CLA; HDL-C, HDL cholesterol; LA, linoleic acid; LDL-C, LDL cholesterol; nonHDL-C, VLDL and LDL cholesterol.

mer. Preliminary evidence from a non-dose–response study from our laboratory would suggest that animals fed mixed CLA isomers had less atherosclerosis than those fed LA (6). However, for reasons mentioned hereafter, these preliminary studies must be interpreted with caution. Also, a recent study (7) showed that CLA feeding promoted fatty streak formation in the C57BL/6 mouse atherosclerosis model. However, this finding may be specific to this animal model and not to others. A study by Lee *et al.* (8) in rabbits showed that at the only concentration studied, 0.5% (w/w), CLA as the FFA reduced aortic atherosclerosis. Recently, a study published by Gavino *et al.* (9) showed that feeding a diet containing an isomeric mixture of CLA significantly reduced plasma TG and total cholesterol levels in hamsters compared with LA and the 9*c*,11*t* CLA isomer. However, their study did not investigate the development of atherosclerosis.

Our recent study in hamsters fed a chow-based diet containing 0.05, 0.1, and 1.0 en% mixed CLA isomers as the FFA also showed an antiatherogenic effect that was associated with increased plasma vitamin E levels (10). Our preliminary study (6) suggested that the FFA was more effective than TG as an antiatherogenic compound. In our earlier mixed-CLA isomer study (10), hamsters were extremely hypercholesterolemic (plasma cholesterol levels >700 mg/dL), raising the question of the efficacy of CLA during more moderate hypercholesterolemia. Also, in that study (10), the hamsters were housed grouped, which may be the reason for the very high levels of blood cholesterol in those animals; in another study in our laboratory, group housing of hamsters was shown to be associated with adverse effects on plasma lipids (11). Data have also indicated that mixed and individual isomers of CLA are effective inhibitors of atherogenesis and also cause regression of established atherosclerosis in rabbits (12–14).

Much of the previous work that has been performed in animals fed CLA has been done using the mixed-isomer formulation of commercially available CLA. The current study examines the purified form of either 9*c*,11*t* or *trans*-10,*cis*-12 (10*t*,12*c*) CLA in the hypercholesterolemic hamster model to determine whether they have similar hypocholesterolemic, glucose hemostatic, and antiatherogenic properties.

EXPERIMENTAL PROCEDURES

Experimental design and diets. One hundred male F_1B strain Syrian Golden Hamsters (*Mesocricetus auratus*) (BioBreeders Inc., Watertown, MA) approximately 9 wk of age were housed in individual stainless steel hanging cages at room temperature with a 12-h light/dark cycle. Hamsters were given food and water *ad libitum*. Animals were fed Purina chow 5001 (Ralston Purina, St. Louis, MO) for a period of 1 wk prior to the start of the study to become acclimated to the facility. All 100 hamsters were fed a chow-based (nonpurified) hypercholesterolemic diet (HCD) containing 10% coconut oil (92% saturated fat) and 0.1% cholesterol for 2 wk. A nonpurified diet, rather than a semipurified diet, was used because published data from our laboratory (15) and those from another laboratory (16) indicated that hamsters on a nonpurified diet were more responsive to various cholesterolemic interventions and a resultant lipoprotein profile [VLDL and LDL cholesterol (nonHDL-C) > HDL cholesterol (HDL-C)] which was similar to that of humans. After an overnight fast, the hamsters were bled and plasma cholesterol concentrations were measured. The hamsters were then divided into 4 groups of 25 based on similar mean plasma nonHDL-C concentrations. Group 1 remained on the HCD (control). Group 2 was fed the HCD plus 0.5% 9*c*,11*t* CLA. Group 3 was fed the HCD plus 0.5% 10*t*,12*c* CLA. Group 4 was fed the HCD plus 0.5% LA. Dietary CLA isomers in pure form and LA were added to the diets as the FFA form and were obtained from Matreya, Inc. (Pleasant Gap, PA). The two CLA isomers and LA were >98% pure by GC. The FA composition of the treatment diets is provided in Table 1. Dietary treatments were fed for 12 wk. Plasma lipids were measured at 0, 8, and 12 wk of dietary treatment. At the end of dietary treatment, the hamsters were sacrificed and aortas were collected. Food disappearance and body weights were measured on a daily basis throughout the study. The animals were maintained in accordance with the guidelines of the Committee on Animal Care of the University of Massachusetts Lowell Research Foundation, as well as the guidelines prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW publication no. 85–23, revised 1985), following approval by the Institutional Animal Care and Use Committee.

TABLE 1
FA Composition of Dietary Treatments (mg/g of diet)

FA	Control	9*c*,11*t* CLA	10*t*,12*c* CLA	LA[a]
8:0	6.7	6.7	6.7	6.7
10:0	5.8	5.8	5.8	5.8
12:0	49.0	49.0	49.0	49.0
14:0	18.3	18.3	18.3	18.3
16:0	8.8	8.8	8.8	8.8
18:0	2.7	2.7	2.7	2.7
18:1	6.7	6.7	6.7	6.7
18:2 (LA[a])	1.5	1.6	1.6	6.5
9*c*,11*t* CLA	—	4.9	—	—
10*t*,12*c* CLA	—	—	4.9	—

[a]LA, linoleic acid.

Plasma lipoprotein cholesterol, TG, and glucose measurements. Blood samples were collected after an overnight fast (16 h). Blood from fasted hamsters, anesthetized with ultrapure 50:50 CO_2/O_2 (Northeast Airgas, Salem, NH), was collected *via* the retro-orbital sinus into heparinized tubes, and plasma was harvested after low-speed centrifugation at 2500 × *g* for 15 min at room temperature. Plasma was frozen at −80°C until analyzed for plasma total cholesterol, HDL-C, nonHDL-C, TG, and glucose concentrations. Plasma cholesterol (17) and TG (18) were measured enzymatically, and after the apoB-containing lipoproteins VLDL and LDL were precipitated with phosphotungstate reagent (19), the supernatant was assayed for HDL-C (Sigma, St. Louis, MO). Plasma nonHDL-C was calculated from the difference between total cholesterol and HDL-C. Plasma lipid determinations are standardized by participa-

tion in the Center for Disease Control–National Heart, Lung, and Blood Institute Standardization Program. Plasma glucose concentrations were also measured enzymatically using Kit #17–100 (Sigma).

Liver lipid measurements. Liver lipid concentrations were measured by a previously described method (20). A 100-mg (wet wt) portion of liver was homogenized with 50 mg of sodium sulfate. Four milliliters of methanol was then added and the tissue was homogenized a second time, followed by addition of 8 mL of chloroform. After mixing, 3 mL of a solution containing 1.25% KCl and 0.05% H_2SO_4 was added and centrifuged at 400 × *g* at room temperature for 10 min. The bottom layer was transferred, and the supernatant was re-extracted with 3 mL of chloroform/methanol (2:1) and centrifuged at 400 × *g* at room temperature for 10 min. The bottom layer was transferred and pooled with the previous supernatant. The solution was placed in a 37°C water bath under N_2. When approximately half of the solution was evaporated, 1 mL of chloroform containing 1% Triton-100 was added, mixed, and evaporated to dryness at 37°C under N_2. Five hundred microliters of distilled water was added to the samples, which were mixed and placed in a shaking water bath at 37°C for 20 min to solubilize the lipid. After incubation, liver total and free cholesterol and phospholipid concentrations were determined enzymatically using Wako total and free cholesterol C and phospholipid kits (Wako Chemicals, Richmond, VA). The hepatic cholesterol ester concentration was determined as the difference between the total and free cholesterol concentrations. Liver TG concentrations were as stated above for plasma TG measurements.

Collection of aortas. At the time of sacrifice (week 12), hamsters were anesthetized with an ip injection of sodium pentobarbital (62.5 mg/mL at a dosage of 0.2–0.25 mL/200 g body weight) (Henry Schein, Port Washington, NY), and aortic tissue was obtained for aortic cholesterol analysis (21). The heart and thoracic aorta were removed and stored in vials containing PBS at 4°C for subsequent analysis. To measure the extent of the aortic cholesterol accumulation in the aortic arch, a piece of thoracic aortic tissue extending from as close to the heart as possible to the branch of the left subclavian artery was used.

Aortic cholesterol measurement. The tissue was weighed and placed in a 25-mL screw-capped test tube (21). Four milliliters of methanol and 8 mL of chloroform were added. After mixing, the solution was allowed to stand at room temperature for 48 h for extraction of cholesterol from the blood vessel wall. After extraction, the aortic tissue was removed and the solution was placed in a 37°C water bath under N_2. When approximately half of the solution was evaporated, 1 mL of chloroform containing 1% Triton-100 was added, mixed, and evaporated to dryness at 37°C under N_2. Two hundred microliters of distilled water was added to the samples, which were mixed and placed in a shaking water bath at 37°C for 20 min to solubilize the lipids. After incubation, aortic total and free cholesterol concentrations were determined enzymatically using Wako total and free cholesterol C kits (Wako Chemicals). The aortic cholesteryl ester concentration was determined as the difference between the total and free cholesterol concentrations. A pilot study was conducted to evaluate the extent to which this procedure removed tissue cholesterol. Aortic cholesterol concentrations were determined after tissue was placed in solvent (4 mL of methanol and 10 mL of chloroform) overnight with frequent vigorous mixing, and were compared with the concentrations obtained following tissue mincing or homogenization as reported previously (22). No significant differences in aortic cholesterol content were observed between the different cholesterol extraction procedures.

Statistical methods. SigmaStat software was used for all statistical evaluations (Jandel Scientific, San Rafael, CA) (23). Differences between time points were determined by using repeated-measures one-way ANOVA followed by Student–Neuman–Keuls *post hoc* test. Differences between the dietary treatments were determined using one-way ANOVA followed by Student–Neuman–Keuls *post hoc* test. All values are expressed as mean ± SD, and significance was set at $P < 0.05$.

RESULTS

All the animals survived the treatment period. As seen in Table 2, there were no differences between any of the animals for initial or final body weights and between food consumption over the course of the study. Also, all the animals in each group gained body weight over the course of the study. Liver weights were significantly greater in the animals fed the 10*t*,12*c* CLA isomer compared with the control (18%), 9*c*,11*t* CLA isomer (14%), and LA (16%) animals ($P < 0.03$) (Table 2).

No significant differences were observed for plasma lipids within dietary treatments between the week 8 and week 12 data. However, statistical differences between groups for plasma lipid measurements were different at 8 and 12 wk; thus, the data have been shown separately. No differences between any of the dietary treatments were observed for plasma total cholesterol, nonHDL-C, and HDL-C concentrations after 8 wk

TABLE 2
Initial and Final Body Weights (g), Food Consumption (g/d), and Organ Weights (g) After 12 wk of CLA or LA Treatment[a]

Diet	Initial body weight	Final body weight	Food consumption	Liver weight	Adipose (perirenal) weight
Control	93.5 ± 8.69	125.5 ± 23.9	14.7 ± 2.20	4.11 ± 0.50[a]	0.91 ± 0.23
0.5% 9*c*,11*t* CLA	91.5 ± 11.7	130.2 ± 24.8	14.9 ± 1.41	4.26 ± 0.66[a]	0.87 ± 0.17
0.5% 10*t*,12*c* CLA	94.7 ± 11.2	129.4 ± 24.5	14.7 ± 1.28	4.86 ± 0.88[b]	0.80 ± 0.20
0.5% LA	95.2 ± 8.20	128.0 ± 27.3	14.9 ± 2.13	4.19 ± 0.63[a]	0.85 ± 0.15

[a]Values are mean ± SD; $n = 25$. Values in a column not sharing a roman superscript are significantly different at $P < 0.05$. For abbreviation see Table 1.

TABLE 3
Plasma Cholesterol, Lipoprotein Cholesterol, TG, and Glucose Concentrations in Hamsters (week 8) (mg/dL)[a]

Diet	TC	nonHDL-C	HDL-C	TG	TC/HDL-C	Glucose
Control	310.0 ± 94.1	241.6 ± 95.4	68.3 ± 3.38	479.5 ± 248.6[a]	4.57 ± 1.45	110.5 ± 18.3[a,b]
0.5% 9*c*,11*t* CLA	268.3 ± 85.5	200.3 ± 86.0	68.1 ± 5.32	562.6 ± 310.9[a]	3.97 ± 1.32	109.0 ± 21.8[a]
0.5% 10*t*,12*c* CLA	288.0 ± 36.3	223.3 ± 38.7	64.8 ± 6.41	794.7 ± 341.4[b]	4.50 ± 0.81	126.1 ± 16.4[a,b]
0.5% LA	260.4 ± 73.4	194.2 ± 73.3	66.2 ± 3.97	483.5 ± 137.0[a]	3.95 ± 1.13	128.5 ± 16.9[b]

[a]Values are mean ± SD; $n = 25$. Values in a column not sharing a roman superscript are significantly different at $P < 0.05$. TC, total cholesterol; nonHDL-C, VLDL and LDL cholesterol; HDL-C, HDL cholesterol; for other abbreviation see Table 1.

TABLE 4
Plasma Cholesterol, Lipoprotein Cholesterol, TG, and Glucose Concentrations in Hamsters (week 12) (mg/dL)[a]

Diet	TC	nonHDL-C	HDL-C	TG	TC/HDL-C	Glucose
Control	316.7 ± 131.6[a]	222.4 ± 152.7[a]	94.4 ± 17.1[a]	413.0 ± 235.7[a]	3.58 ± 2.14	115.3 ± 15.3[a]
0.5% 9*c*,11*t* CLA	203.1 ± 29.0[b]	124.3 ± 31.6[b]	78.8 ± 9.57[b]	199.0 ± 39.9[b]	2.62 ± 0.51	99.4 ± 13.5[a]
0.5% 10*t*,12*c* CLA	205.5 ± 31.9[b]	133.8 ± 34.1[b]	71.8 ± 7.23[b]	304.4 ± 80.6[a,b]	2.90 ± 0.59	153.3 ± 34.6[b]
0.5% LA	227.5 ± 46.8[b]	157.9 ± 45.1[a,b]	69.5 ± 7.78[b]	291.5 ± 106.0[a,b]	3.29 ± 0.67	129.9 ± 16.9[b]

[a]Values are mean ± SD; $n = 25$. Values in a column not sharing a roman superscript are significantly different at $P < 0.05$. For abbreviations see Tables 1 and 3.

of dietary treatment (Table 3). Plasma TG, however, were significantly higher in the hamsters fed the 10*t*,12*c* CLA isomer compared with hamsters fed the control (40%), 9*c*,11*t* CLA isomer (29%), and LA (39%) diets after 8 wk of treatment ($P < 0.004$). Also, hamsters fed the LA diet had significantly higher plasma glucose concentrations compared with hamsters fed the 9*c*,11*t* CLA isomer (15%) after 8 wk of treatment ($P < 0.001$).

After 12 wk of dietary treatment, plasma total cholesterol and HDL-C concentrations were significantly lower in the hamsters fed the 9*c*,11*t* CLA isomer (−35 and −17%, respectively), 10*t*,12*c* CLA isomer (−35 and −24%, respectively), and LA (−28 and −26%, respectively) diets compared hamsters fed the control diet ($P < 0.001$) (Table 4). Plasma nonHDL-C concentrations were significantly lower in hamsters fed the 9*c*,11*t* CLA isomer (−44%) and the 10*t*,12*c* CLA isomer (−40%) diets but not in those fed the LA diet, compared with hamsters fed the control diet after 12 wk of treatment ($P < 0.001$). Plasma TG concentrations were significantly lower in the hamsters fed the 9*c*,11*t* CLA isomer compared with those fed the control (−52%) diet after 12 wk of treatment ($P < 0.03$). Also, hamsters fed the 10*t*,12*c* CLA isomer and the LA diets had significantly higher plasma glucose concentrations compared with those fed the control (25 and 11%, respectively) and 9*c*,11*t* CLA isomer (35 and 23%, respectively) diets after 12 wk of treatment ($P < 0.008$).

Although liver weights were significantly higher in hamsters fed the 10*t*,12*c* CLA isomer diet, liver total cholesterol, free cholesterol, cholesterol ester, and TG concentrations were significantly lower in these hamsters than in those fed the control (−51, −28, −59, and −45%, respectively), 9*c*,11*t* CLA isomer (−53, −33, −60, and −46%, respectively), and LA (−47, −30, −53, and −40%, respectively) diets ($P < 0.05$) (Table 5). None of the other treatment groups were significantly different from each other for these same variables. Also, no treatment groups were significantly different from each other for liver phospholipid concentrations (Table 5).

Table 6 shows the data for aortic cholesterol accumulation measurements after 12 wk of dietary treatment. Although there were no statistically significant differences between any of the dietary treatments for aortic cholesterol accumulation, a small trend was observed. The 9*c*,11*t* CLA isomer tended to reduce total cholesterol and cholesteryl ester accumulation in the aortic arch compared with the 10*t*,12*c* CLA isomer (−18 and −13%, respectively, $P = 0.11$), but not significantly.

DISCUSSION

Our results indicate that supplementing the diets of hypercholesterolemic hamsters with individual CLA isomers reduces plasma cholesterol concentrations when compared with controls, without any changes in body weight and aortic cholesterol accumulation. The effects of CLA on body weight and composition have been controversial in both animal and human studies. In the current study, no differences in final body

TABLE 5
Liver TC, Free Cholesterol (FC), Cholesterol Ester (CE), TG, and Phospholipid (PL) Concentrations in Hamsters (mg/g liver tissue)[a]

Diet	TC	FC	CE	TG	PL
Control	11.15 ± 3.93[a]	2.99 ± 0.76[a]	8.16 ± 3.47[a]	6.29 ± 2.98[a]	14.89 ± 5.44
0.5% 9*c*,11*t* CLA	11.50 ± 5.33[a]	3.20 ± 0.86[a]	8.30 ± 4.56[a]	6.35 ± 2.63[a]	15.60 ± 3.53
0.5% 10*t*,12*c* CLA	5.46 ± 0.88[b]	2.14 ± 0.59[b]	3.33 ± 2.31[b]	3.43 ± 1.28[b]	12.72 ± 3.23
0.5% LA	10.22 ± 3.68[a]	3.07 ± 0.82[a]	7.15 ± 3.11[a]	5.76 ± 2.16[b]	15.48 ± 3.68

[a]Values are mean ± SD; $n = 25$. Values in a column not sharing a roman superscript are significantly different at $P < 0.05$. For other abbreviations see Tables 1 and 3.

TABLE 6
Aortic Cholesterol Concentrations (μg/mg of tissue)[a]

Diet	FC	CE	TC
Control	1.96 ± 1.48	0.80 ± 0.45	2.76 ± 1.36
0.5% 9*c*,11*t* CLA	1.93 ± 1.54	0.74 ± 0.54	2.63 ± 1.37
0.5% 10*t*,12*c* CLA	2.13 ± 1.65	0.90 ± 0.53	3.03 ± 1.75
0.5% LA	1.91 ± 1.33	0.79 ± 0.60	2.71 ± 1.53

[a]Values are mean ± SD, $n = 25$. For abbreviations see Tables 1, 3, and 5.

weights were observed between hamsters fed the control diet or diets supplemented with CLA isomers or LA. Previous work in young rats fed diets supplemented with 0.5% CLA showed, in animals fed the CLA, an increase in body weight without changes in food consumption compared with controls (24). In contrast to this study, Park *et al.* (25) reported in mice that the effects of CLA on body composition changes appeared to be due in part to reduced fat deposition and increased lipolysis in adipocytes. Animal studies in general have suggested that the 10*t*,12*c* isomer, and not the 9*c*,11*t* isomer, has the most potent body fat-reducing properties (9,25). In the current study, no differences were observed for perirenal adipose tissue weight. However, the 10*t*,12*c* CLA isomer did reduce it slightly compared with the control. One possible explanation for the nonsignificant effect of either CLA isomer on the reduction of fat tissue is that the effects of CLA on the perirenal depot are more sensitive when diets are low in fat (4%), but not when diets are high in fat (10%) (26), as in the current study. In humans, the majority of studies have shown no effect on body weight or composition when individual or mixed isomers of CLA were fed (27–31). However, one recent long-term trial (1 yr) showed that CLA feeding decreased body fat mass without any adverse effects (32). Other differences between earlier studies that showed positive effects and this study on body weight changes may be due to feeding the mixed CLA isomer in the earlier studies compared with feeding the individual isomers in the current study, or to species differences. Since previous work in hamsters fed a mixed-isomer diet showed significantly less weight gain than did either the 9*c*,11*t* isomer- or LA-supplemented groups (9) and showed that the mixed-isomer diet decreased fat depot weights compared with the 9*c*,11*t* isomer diet in other studies (25,33), there may be synergistic effects on body weight or composition when both the 9*c*,11*t* and 10*t*,12*c* CLA isomers are supplemented simultaneously. Overall, however, there is no conclusive evidence to suggest that consumption either of mixtures of CLA isomers or of highly enriched preparations of individual isomers results in a significant reduction in body composition in animals, including humans.

Some studies have shown that feeding only the 10*t*,12*c* CLA isomer is associated with greater liver weights in hamsters and with a significant decrease or no change in the amount of TG accumulation in the liver (33–35). However, other studies in mice have shown the same hepatomegoly with mixed CLA isomer feeding, and this was associated with greater TG accumulation (36–39). In the current study, larger livers were also found in hamsters fed the 10*t*,12*c* CLA isomer but not in hamsters fed the 9*c*,11*t* CLA isomer or LA. Similar to previous work in hamsters (35), this increase in liver weights in hamsters fed the 10*t*,12*c* CLA isomer was not due to increased lipid accumulation. In fact, levels of liver TG, cholesterol esters, and free cholesterol were significantly decreased in these hamsters compared with the effects of other dietary treatments. Differences between the current study and those by others in which an increase in lipid accumulation was observed (36–39) may be due to the feeding of individual isomers vs. mixed CLA or due to different species (mice vs. hamsters) being studied. However, it is still unknown what may have led to the increase in liver weights in hamsters fed the 10*t*,12*c* CLA isomer in the current study, but it could be due to an increase in the total number of hepatocytes with the 10*t*,12*c* CLA isomer, as previously observed (35).

Previous studies from our laboratory (6,10) and from others (8,9) have shown reductions in plasma cholesterol concentrations in mixed CLA-fed animals compared with controls. The results of the current study with the individual CLA isomer formulations compared with controls are consistent with these previous studies (40) for plasma cholesterol lowering. However, in the current study we observed no significant differences between feeding the individual CLA isomers and LA for plasma cholesterol lowering, whereas an earlier study from our laboratory did show that mixed CLA feeding produced significantly lower plasma cholesterol levels compared with LA (10), but another did not (6). Presently, there is no explanation for this discrepancy, although the difference may be due to the different diets that were used in these studies. In the present study, we fed the different isomers individually in the diets, compared with feeding a mixed CLA isomer formulation in the earlier study (10). Previous work (9) showed that the 9*c*,11*t* CLA isomer had no effect on plasma cholesterol concentrations compared with control hamsters. However, in that study (9) only 0.2% of the 9*c*,11*t* CLA isomer was fed to hamsters, whereas in the current study 0.5% was fed to hamsters. Another study (40) also showed a reduction in plasma cholesterol concentrations with both the 9*c*,11*t* and 10*t*,12*c* CLA isomers and with LA when compared with control hamsters. Similar contradictory results have been observed in human studies. Tricon *et al.* (27) showed a significant effect of isomer supplementation on blood cholesterol concentrations in humans. Their data suggest a trend toward a decrease in plasma cholesterol and LDL-C concentrations after supplementation with the 9*c*,11*t* CLA isomer, but not the 10*t*,12*c* CLA isomer (27). Other studies have also shown a cholesterol-lowering effect of CLA in humans

(30,41). However, more studies appear to have shown no effect on blood cholesterol levels in humans when fed CLA (31,42,43). The observation in the present study that individual or mixed CLA isomer feeding reduced plasma HDL-C is similar to some previous studies in animals (31,36) and humans (44), but not to others (6,9,10,27,42,43).

We also observed an increase in plasma TG when feeding the 10*t*,12*c* CLA isomer compared with the other treatments after 8 wk of supplementation, but not after 12 wk. By 12 wk of treatment, the 9*c*,11*t* CLA isomer had significantly lowered plasma TG compared with hamsters fed the control. These results are similar to our previous study (6), which showed that feeding mixed-isomer CLA increased plasma TG more than did LA. In contrast, previous studies in hamsters (9,10) and humans (29) have shown that feeding the mixed CLA either reduced or had no effect on plasma TG concentrations. The differences between those studies and the current study may be due to the different concentrations of purified isomers used in these studies or to the use of mixed CLA isomers vs. individual isomers. However, one study (27) in healthy humans showed that plasma TG concentrations were higher during supplementation with the 10*t*,12*c* CLA isomer than during supplementation with the 9*c*,11*t* CLA isomer. The TG-raising potential of mixed CLA may be predominantly due to the 10*t*,12*c* isomer, as shown in the current study and in others (27). Along with the slight changes in plasma TG concentrations with the different CLA isomers, we also observed changes in plasma glucose concentrations. In the current study, the 9*c*,11*t* CLA isomer resulted in significantly lower plasma glucose concentrations compared with the 10*t*,12*c* CLA isomer and LA diets, similar to previous studies in humans (27,44). Risérus *et al.* (44) also showed that feeding the 9*c*,11*t* CLA isomer decreased insulin resistance whereas feeding the 10*t*,12*c* CLA isomer increased insulin resistance in obese men. Also, a study by Moloney *et al.* (45) showed that supplementation with mixed CLA significantly increased fasting glucose concentrations. In contrast, CLA feeding was shown to improve glucose concentrations in type 2 diabetics (46) and in rats (47). The difference between these studies (46,47) and the current one may again be due to the use of mixed CLA vs. individual isomers, or to species differences.

The present study also showed that feeding CLA as individual isomers did not produce a significant change in early aortic atherosclerosis, as measured by aortic cholesterol accumulation, compared with both LA and the control diet, a result that is inconsistent with previous work (9,10). However, this observation in the former study (10) was not significant until the data for the three different doses of CLA feeding were combined and then compared with the HCD-fed animals; also, the CLA used for the earlier studies was mixed CLA. Additionally, in our earlier study (10), the hamsters that were fed CLA had a significantly greater plasma tocopherol/total cholesterol ratio than did hamsters fed a control diet or LA-containing diet. These results suggest that CLA may be tocopherol sparing and thus act directly or indirectly as an antioxidant *in vivo*, which may contribute to its antiatherogenic properties. Another possible explanation for the discrepancy is that earlier studies have either used fatty streak area (10) or visual staining procedures (15), measures of early atherosclerosis that may not be as appropriate as the biochemical measurement of aortic cholesterol accumulation used in the current study. Recently, Mitchell *et al.* (40) showed a similar result when feeding the individual isomers compared with LA, in that there were no significant differences in aortic fatty streak lesions but that a slight decrease was observed with the CLA isomers.

Although, in the current study, feeding LA to hamsters produced a significant decrease in plasma total cholesterol and HDL-C concentrations, similar to CLA feeding, it did not produce a significant reduction in nonHDL-C concentrations, as did the CLA isomers after 12 wk of dietary treatment or in early atherosclerosis. This nonsignificant reduction in aortic atherosclerosis with LA feeding in the present study may be due to an increase in LDL oxidative susceptibility that is associated with LA feeding, which was not measured. Previous studies have shown that LDL particles enriched with LA are more susceptible to oxidation and presumably more atherogenic than are oleate-rich diets (3,4).

In summary, both CLA isomers produced significant lowering in plasma total cholesterol and nonHDL-C concentrations; however, the majority of human studies have shown no effect. Also, no significant differences were observed between the CLA isomers (9*c*,11*t* vs. 10*t*,12*c*) for changes in plasma lipids or lipoprotein cholesterol concentrations; however, the 10*t*,12*c* CLA isomer did raise plasma glucose concentrations compared with the 9*c*,11*t* CLA isomer, and only the 9*c*,11*t* CLA isomer reduced plasma TG compared with the control. The lower plasma glucose concentrations, along with the lower plasma TG concentrations, may decrease the risk of insulin resistance and type 2 diabetes in humans when the 9*c*,11*t* CLA isomer is fed alone.

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Dietary Comparison of Conjugated Linolenic Acid (9 *cis*, 11 *trans*, 13 *trans*) and α-Tocopherol Effects on Blood Lipids and Lipid Peroxidation in Alloxan-Induced Diabetes Mellitus in Rats

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ABSTRACT: The present study investigated the dietary effect of conjugated linolenic acid (CLnA) on lipid profiles and lipid peroxidations in alloxan-induced diabetes mellitus in rats. Diabetic rats were fed with 20% sunflower oil (diabetic control), sunflower oil supplemented with 0.5% CLnA, sunflower oil supplemented with 0.15% α-tocopherol, and sunflower oil containing 0.25% CLnA + 0.15% α-tocopherol. The results demonstrated that 0.5% CLnA, 0.15% α-tocopherol, and 0.25% CLnA + 0.15% α-tocopherol each on supplementation significantly lowered total cholesterol and non-HDL-cholesterol in comparison with the diabetic control group. The TAG level was significantly lowered in both the 0.15% α-tocopherol and 0.25% CLnA + 0.15% α-tocopherol groups. LDL-lipid peroxidation and erythrocyte membrane lipid peroxidation were reduced significantly in each of the experimental groups vs. the control group. The CLnA + α-tocopherol diet induced a greater reduction in membrane lipid and liver lipid peroxidation than the α-tocopherol diet alone. In conclusion, dietary CLnA exerts antioxidant activity as evidenced by reduced lipid peroxidation in chemically induced diabetes mellitus.

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Oxidant free radicals have been implicated in the pathogenesis of type I diabetes mellitus (DM) (1,2). In addition, diabetic patients have significant defects of antioxidant protection (3,4). Glucose auto oxidation, polyol pathway, and protein glycation are biochemical pathways associated with hyperglycemia and toxic superoxide intermediates (4). DM is also associated with the peroxidation of lipids and plasma lipoproteins (5). Oxidized lipoproteins lead to the development of atherosclerosis and diabetic vascular complications.

Antioxidants scavenge free radicals and reduce the deleterious consequences within the lipid. Conjugated linoleic acid (CLA) has been proved to prevent noninsulin-dependent DM (6). Garlic oil (7), fenugreek (8), and ferulic acid (9) each act as an antioxidant and reduce pathological conditions of DM in rats.

Conjugated linolenic acid (CLnA) (α-eleostearic acid: 9*c*,11*t*,13*t*-18:3), commonly found in karela seed (*Momordica charantia*), was evaluated nutritionally in a previous study (10). When CLnA was fed as dietary FA to rats at 0.5, 2, and 10% levels with sunflower oil, CLnA significantly lowered plasma lipid peroxidation, erythrocyte membrane (EM) lipid peroxidation, and liver tissue lipid peroxidation in comparison with the sunflower oil group at a 0.5% level (11). The antioxidant property decreased with the increase of CLnA level in the diet. Koba *et al.* (12) reported significant effects of dietary CLnA on body fat, serum lipid, and liver lipid levels in rats. A cancer chemoprotective activity in the early phase of colon tumorigenesis has also been reported (13).

The aim of the present study was to examine the antioxidant activity of CLnA, in the absence and presence of α-tocopherol as a protective agent against atherosclerosis and DM.

EXPERIMENTAL PROCEDURES

Dietary fat sources. Oil was extracted from authentic karela (bitter gourd) seed, obtained from the local market in Calcutta, India, in a Soxhlet apparatus with food-grade *n*-hexane (40–60°C boiling range). The FFA present in the oil were then removed by the miscella refining process (14). The extracted oil containing hexane, known as miscella (hexane/oil 2:1, vol/wt) was mixed with 10% NaOH solution (20% excess of the theoretical amount required) to neutralize the FFA at 40°C for 30 min. 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 bleached with Tonsil earth (1% w/w) obtained from P.T. Sud-Chemical (Jakarta, Indonesia) and activated carbon (2% w/w), supplied

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Abbreviations: CLA, conjugated linoleic acid; CLnA, conjugated linolenic acid; DC, diabetic control group (group of rats feeding on sunflower oil under diabetic conditions); D-CLnA, diabetic conjugated linolenic acid group (group of rats feeding on sunflower oil containing 0.5% CLnA under diabetic conditions); DM, diabetes mellitus; D-Toc, diabetic tocopherol group (group of rats feeding on sunflower oil containing 0.15% α-tocopherol under diabetic conditions); D-Toc-CLnA, diabetic tocopherol and conjugated linolenic acid group (group of rats feeding on sunflower oil containing 0.15% α-tocopherol + 0.25% CLnA under diabetic conditions); EM, erythrocyte membrane; LA, linoleic acid; LOS, lipoprotein oxidation susceptibility; MDA, malondialdehyde; NDC, nondiabetic control group (group of rats feeding on sunflower oil under normal conditions); RBC, red blood cell; TBA, thiobarbituric acid.

TABLE 1
FA Composition of Dietary Oils and Oil Mixtures

	FA composition (area%)				
Dietary fats	16:0	18:0	18:1	18:2	18:3 (CLnA, 9*c*,11*t*,13*t*-18:3)
Sunflower oil	6.2	3.4	32.8	57.6	—
Karela seed oil	1.4	31.5	6.2	3.2	57.7
Sunflower oil + karela seed oil (99:1, w/w)	6.1	3.9	32.4	57.1	0.5
Sunflower oil + karela seed oil (99.5:0.5, w/w)	6.1	3.9	32.55	57.2	0.25

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 –40°C under nitrogen.

Dietary fat blends. Sunflower oil was obtained from I.T.C. Limited (Hyderabad, India). Sunflower oil was mixed with karela seed oil, α-tocopherol, and a mixture of karela seed oil and α-tocopherol to give final oil mixtures containing 0.5% by weight of CLnA, 0.15% α-tocopherol, and 0.15% α-tocopherol + 0.25% CLnA; see Table 1 for FA composition of dietary oil mixtures.

Analysis of fat products. GC (Hewlett-Packard 5890A) was used to determine FA composition (11). The chromatographic conditions were: detector, FID; carrier gas, nitrogen; flow rate, 30 mL/min; column, 10% diethylene glycol succinate, $6' \times 1/8''$ (1800×3 mm) i.d. The oven, injection port, and detector block temperatures were 190, 230, and 240°C, respectively. The sample of methyl ester (0.1–1 μg) was injected with a Hamilton syringe and the chromatogram was obtained. The identifications of FA were made by comparison with the retention times of GLC-grade standard methyl esters of varying chain lengths and unsaturations. The percent compositions of the component FA were determined by measuring the areas of the peaks with the help of the integrator (Hewlett-Packard model 3390A).

Preparation of methyl ester. About 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 after the addition of 1.0 mL of 1 N HCl was then shaken vigorously. Methyl esters were extracted with 3×1 mL portions of petroleum ether. The extracts were combined in a screw-capped test tube, and the solvent was removed by a flow of nitrogen.

Conjugation of α-eleostearic acid present in karela seed oil was determined by UV spectrometric analysis at 262, 268, and 274 nm (15).

Feeding experiment. Male albino rats of Charles Foster strain (selected for the authenticity of the strain) were housed in individual cages. The work was done under the supervision of the Animal Ethical Committee of the Department of Chemical Technology (University of Calcutta). The animals were divided into five groups (average body weight 70–80 g), each consisting of eight animals. Diabetes mellitus was induced in control and experimental groups of rats (after attaining their average body weight 100 g) by a single intraperitoneal injection of 200 mg alloxan/kg body weight, freshly prepared in sterilized water (7). The rats with blood sugar levels >230 mg/dL, indicating glycemia, were considered diabetic. The first group was the nondiabetic control group (NDC) without any treatment. The second group received sunflower oil and served as the diabetic control group (DC). The third, fourth, and fifth groups received sunflower oil along with karela seed oil (D-CLnA), α-tocopherol (D-Toc), and α-tocopherol + karela seed oil (D-Toc-CLnA), respectively.

The rats were fed experimental diets having the following composition: 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 (16)]; cellulose, 3%; and one multivitamin capsule (vitamin A I.P. 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, and 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 6 wk. The amount of daily diet consumed by each rat and weekly body weight gain were recorded. Rats were fasted overnight for 12 h and then sacrificed under anesthesia; blood was collected, and liver and heart were immediately excised, blotted, and stored frozen (–40°C) for analysis.

Lipid analysis. According to the standard methods, the lipid components such as total cholesterol (17), and HDL-cholesterol (18) of plasma were analyzed using enzyme kits supplied by Ranbaxy Diagnostics Ltd. (New Delhi, India).

Plasma lipid peroxidation was measured by the assay of TBARS according to the standard method (19). The amount of malondialdehyde (MDA) formed was calculated by taking the extinction coefficient of MDA to be $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Lipoprotein oxidation susceptibility (LOS) test. A 500-μL plasma sample was treated with 50 μL of a solution containing 0.2 mM dextran sulfate (M.W. 50,000; Genzyme, Cambridge, MA) and 0.5 M $MgCl_2 \cdot 6H_2O$ to precipitate the apolipoprotein B-containing lipoproteins (LDL and VLDL) according to Bachorik and Albers (20). After centrifugation at $3000 \times g$ at 20°C for 10 min, the supernatant was removed, and 1 mL of 6% BSA and another 50 μL of the dextran sulfate solution was added. The solution was briefly vortexed and recentrifuged as above

to wash away any HDL or residual serum proteins (except, of course, albumin). The supernatant was removed, and washed precipitate (containing LDL and VLDL) was dissolved in 2.5 µL of 4% NaCl. A volume of redissolved precipitate containing 100 µg of non-HDL-cholesterol was combined with sufficient 4% NaCl to give a total volume of 500 µL (approximately a 1:5 dilution). Fifty microliters of a 0.5 mM $CuCl_2 \cdot 2H_2O$ solution was added (final copper concentration was 46 µM), and then the samples were incubated at 37°C in a shaking water bath for 3 h. Next TBARS was measured by adding 2 mL of the TBARS reagent to each tube. The mixture was heated at 100°C in a water bath for 15 min. After removing and cooling the tubes, 2.5 mL *n*-butanol was added, the tubes were vortexed and then centrifuged for 15 min at 3000 × *g* at room temperature. The pink upper layer was removed and the O.D. was determined in a spectrophotometer at 532 nm according to the method described by Phelps and Harris (21).

Preparation and oxidative sensitivity of EM ghost (22). After plasma separation, the red blood cells (RBC) were washed three times by centrifugation at 3000 × *g* for 10 min with 3 vol of a cooled isotonic solution containing 0.15 M NaCl and 10^{-5} M EDTA. RBC were hemolyzed using hypotonic solution and centrifuged at 20,000 × *g* for 40 min at 4°C. The supernatant was removed carefully with a Pasteur pipette. The process was repeated two more times. After the last wash step, the supernatant was removed as much as possible and the loosely packed milky-looking membrane pellet was resuspended at the bottom of the tube using 0.89% NaCl solution. The concentrated membrane solution was taken into a 2 mL screw-capped vial and stored at –40°C.

A modification of the 2-thiobarbituric acid (TBA) test (23) was used to measure the lipid peroxides. A 0.5 mL aliquot of the red blood cell membrane suspension was mixed with 1.0 mL of 10% TCA and 2.0 mL of 0.67% 2-TBA. 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 spectrophotometer (Shimadzu, Tokyo, Japan), and the relative amounts of lipid peroxides were expressed in absorbance units, A_{534} nm (24).

Liver tissue lipid extraction and peroxidation. For lipid peroxide measurement, approximately 1 g of liver tissue was placed into a glass centrifuge tube (70 mL) for 2 min in a solvent mixture consisting of 10 mL of chloroform and 20 mL of methanol, and homogenized on ice. Then, 10 mL of chloroform was added and homogenization continued for another 30 s. Finally, 10 mL of redistilled water was added and the mixture was homogenized for 30 s. The tubes were then centrifuged for 20 min at 4000 × *g*, and the chloroform layer was separated in a separatory funnel. A TBA test in chloroform phase from a Bligh and Dyer extraction (25) was performed according to the method described by Schmedes and Hølmer (26). Chloroform (2.5 mL) from the Bligh and Dyer extraction was pipetted into a 30-mL autoclavable glass culture tube (Kimax; Kimble/Kontes, Vineland, NJ) having a Teflon-lined screw cap; 4 mL of the TBA reagent was added and the tube was capped tightly. Safety-shielded tubes were heated for 30 min in a boiling water bath and cooled in tap water. After cooling, 3.5 mL TCA (5%) was added to each tube, mixed thoroughly, and the tubes were then centrifuged at 3000 × *g*.

Statistical analysis. The data were expressed as mean ± SD. A one-way ANOVA was also used for statistical analysis between groups. The *F* ratio of one-way ANOVA is significant when the *P* value < 0.05. Tukey's multiple range method (27) was used for comparison. The statistical program was Minitab release 13.31 (Minitab, State College, PA).

RESULTS

The FA composition of the dietary oils and oil blends is given in Table 1. Oleic (18:1) and linoleic (18:2) acid contents are generally similar in content for the control and experimental dietary fats.

The plasma total cholesterol, HDL-cholesterol, non-HDL-cholesterol, TAG, and blood sugar level of the rats (NDC and DC groups), raised on sunflower oil and sunflower oil containing 0.5% CLnA (D-CLnA group), 0.15% α-tocopherol (D-Toc group), and 0.15% α-tocopherol + 0.25% CLnA (D-CLnA-Toc group) diets, were quantified (Table 2). Diabetes induced by alloxan increased total cholesterol, HDL-cholesterol, non-HDL-cholesterol, TAG, and blood sugar levels significantly in comparison with the corresponding values of the control nondiabetic rats. Plasma total cholesterol in each of the experimental

TABLE 2
Plasma Lipid Profile and Blood Sugar Level of Rats in Different Groups[a,b]

Groups	Total cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	Non-HDL cholesterol (mg/dL)	TAG (mg/dL)	Blood sugar (mg/dL)
Control (nondiabetic, NDC)	63.75 ± 3.25	34.51 ± 2.99	22.368 ± 0.53	55.08 ± 5.89	81.13 ± 4.69
Diabetic control (DC)	83.5 ± 6.18[a]	46.38 ± 5.65	33.62 ± 4.31[a]	86.5 ± 11.33[a]	253.7 ± 22.36[a]
0.5% CLnA (D-CLnA)	66.16 ± 1.57[b]	47.89 ± 6.68	18.91 ± 3.31[b]	69.42 ± 5.73	223.7 ± 12.65
0.15% Tocopherol (D-Toc)	58.19 ± 3.58[c]	46.29 ± 4.5	19.54 ± 3.09[c]	50.38 ± 5.5[c]	206.4 ± 17.06
0.15% Tocopherol + 0.25% CLnA (D-Toc-CLnA)	64.04 ± 3.84[d]	39.35 ± 2.96	20.73 ± 1.75[d]	41.83 ± 8.53[d]	202.58 ± 9.45

[a]NDC, nondiabetic control group fed sunflower oil; DC, diabetic control group fed sunflower oil; D-CLnA, diabetic group fed sunflower oil containing 0.5% CLnA; D-Toc, diabetic group fed sunflower oil containing 0.15% α-tocopherol; D-Toc-CLnA, diabetic group fed sunflower oil containing 0.15% α-tocopherol + 0.25% CLnA.
[b]All values are means of 8 rats/diet. [a]NDC vs. DC; [b]DC vs. D-CLnA; [c]DC vs. D-Toc; [d]DC vs. D-Toc-CLnA. Significant *F* ratios for total cholesterol (*P* value: 0.002), non-HDL-cholesterol (*P* value: 0.009), TAG (*P* value: 0.004), blood sugar (*P* value: 0.0001).

TABLE 3
Plasma Lipid Peroxidation and Lipoprotein Peroxidation of Rats of Different Groups[a,b]

Groups	Plasma peroxidation (nmol of MDA/mL of plasma)	LDL peroxidation (nmol of MDA/mg of non-HDL-cholesterol)
Control (NDC)	2.89 ± 0.62	0.239 ± 0.011
Diabetic control (DC)	14.00 ± 1.11[a]	0.567 ± 0.353[a]
0.5% CLnA (D-CLnA)	12.07 ± 1.55	0.18 ± 0.05
0.15% Tocopherol (D-Toc)	11.09 ± 1.43	0.16 ± 0.04
0.15% Tocopherol + 0.25% CLnA (D-Toc-CLnA)	13.39 ± 1.93	0.061 ± 0.174

[a]MDA, malondialdehyde; for other abbreviations see Table 2.
[b]All values are means of 8 rats/diet. [a]NDC vs. DC. Significant *F* ratios for plasma peroxidation (*P* value: 0.0001). No significant *F* ratio for LDL peroxidation, *F* < critical *F*.

groups was significantly lower than that of the diabetic control (DC) sunflower oil group. There was also a significant lowering of the plasma non-HDL-cholesterol levels in all three experimental groups compared with the DC group. The TAG level was significantly lowered in the D-Toc and D-Toc-CLnA groups.

Plasma lipid peroxidation and LOS induced by copper were measured in the five dietary groups (Table 3). Plasma lipid peroxidation and LDL peroxidation both increased significantly in the DC group compared with the NDC group. No significant differences were noted between the experimental groups and the DC group in the *in vitro* plasma lipid peroxidation. There was significant lowering of LOS in the three experimental diabetic groups of rats when compared with that of the DC group.

The lipid profiles and the extent of lipid peroxidation in EM ghost of the five dietary groups of rats were analyzed (Table 4). Phospholipid and cholesterol contents in the DC group did not increase significantly compared with the NDC group. However, EM lipid peroxidation increased significantly in the DC group. The cholesterol and phospholipid contents showed no significant difference in the experimental dietary groups of rats and the DC group. But TBARS production in EM ghosts of D-CLnA, D-Toc, and D-Toc-CLnA groups was significantly lower than the control DC group without CLnA.

In the case of liver lipids (Table 5), no significant difference was noted in the content of total lipid, total cholesterol, and phospholipid between the NDC group and the DC group. But TAG content and liver lipid peroxidation increased significantly in the DC group compared with the NDC group. The production of TBARS *in vitro* in liver lipid of the D-Toc-CLnA group decreased significantly compared with the DC group. CLnA + α-tocopherol diet decreased liver lipid peroxidation significantly more than α-tocopherol alone.

DISCUSSION

Antioxidative defense systems against oxidative stress, which is related to various degenerative diseases such as DM, cardiovascular disease, arthritis, and aging, are well documented (28). In alloxan-induced DM, high levels of free radicals are generated (29) and cause oxidative damage. Endogenous antioxidants become important in the defense against oxidative damage. We previously reported that CLnA present in karela seed oil has scavenging action against lipid peroxidation (11) and that 0.5% CLnA in the dietary lipid was the optimal dose for the antioxidative effect, as was used also in the current study. Individually, α-tocopherol and CLnA evoked similar antioxidative responses at 0.5 and 0.15% dosage levels, respectively. In the present study, the effect of lipid profiles and lipid peroxidations was examined by reducing the amount of dietary CLnA from 0.5 to 0.25% without changing the dose of α-tocopherol.

Besides changing the blood sugar level, diabetes induced by alloxan changed lipid profiles dramatically. Unlike dietary CLA that significantly increased fasting glucose as demonstrated by Moloney *et al.* (30), dietary CLnA did not increase

TABLE 4
Lipid Profile and Lipid Peroxidation of Erythrocyte Membrane (EM) Ghosts of Rats[a,b]

Diets	Phospholipid (mg/mg of protein)	Cholesterol (mg/mg of protein)	Phospholipid/ cholesterol ratio	EM lipid peroxidation (nmol of MDA/mg of protein)
Control (nondiabetic, NDC)	1.631 ± 0.19	0.34 ± 0.02	4.47 ± 0.57	5.225 ± 0.396
Diabetic control (DC)	1.71 ± 0.24	0.45 ± 0.08	3.84 ± 0.27	12.64 ± 1.78[a]
0.5% CLnA (D-CLnA)	1.45 ± 0.173	0.33 ± 0.04	4.49 ± 0.49	4.78 ± 1.29[b]
0.15% Tocopherol (D-Toc)	1.62 ± 0.18	0.41 ± 0.04	4.04 ± 0.53	7.55 ± 1.67[c]
0.15% Tocopherol + 0.25% CLnA (D-Toc-CLnA)	2.09 ± 0.9	0.33 ± 0.023	4.71 ± 0.5	5.69 ± 0.68[d]

[a]For other abbreviations see Table 2.
[b]All values are means of eight rats/diet. [a]NDC vs. DC; [b]DC vs. D-CLnA; [c]DC vs. D-Toc; [d]DC vs. D-Toc-CLnA. Significant *F* ratios for EM peroxidation (*P* value: 0.001). *F* < critical *F* for phospholipid, cholesterol, phospholipid/cholesterol ratio.

TABLE 5
Liver Lipid Profile and Lipid Peroxidation of Rats[a,b]

Groups	Total lipid (g/g of tissue)	Total cholesterol (mg/g of tissue)	Phospholipid (mg/g of tissue)	TAG (mg/g of tissue)	Liver lipid peroxidation (nmol of MDA/mg of tissue lipid)
Control (nondiabetic, NDC)	0.08 ± 0.01	2.08 ± 0.36	16.01 ± 0.68	6.74 ± 0.57	0.85 ± 0.06
Diabetic control (DC)	0.06 ± 0.02	2.49 ± 0.14	15.78 ± 1.18	12.31 ± 1.61[a]	1.49 ± 0.25[a]
0.5% CLnA (D-CLnA)	0.08 ± 0.02	2.06 ± 0.26	16.27 ± 1.53	6.94 ± 0.78[b]	1.24 ± 0.30
0.15% Tocopherol (D-Toc)	0.07 ± 0.02	3.07 ± 0.38	14.46 ± 1.57	7.7 ± 0.51[c]	1.2 ± 0.17
0.15% Tocopherol + 0.25% CLnA (D-Toc-CLnA)	0.07 ± 0.02	2.30 ± 0.37	16.19 ± 1.25	12.22 ± 1.45	0.54 ± 0.06[d,e]

[a]For abbreviations see Tables 2 and 4.
[b]All values are means of 8 rats/diet. [a]NDC vs. DC; [b]DC vs. D-CLnA; [c]DC vs. D-Toc; [d]DC vs. D-Toc-CLnA;, [e]D-Toc vs. D-Toc-CLnA. Significant *F* ratios for liver lipid peroxidation (*P* value: 0.029), liver TAG (*P* value: 0.001). *F* < critical *F* for liver total lipid, phospholipids, and cholesterol.

blood sugar levels in diabetic rats. The total HDL-cholesterol concentration did not change with dietary CLnA, but CLnA significantly reduced non-HDL-cholesterol such that the HDL/non-HDL cholesterol ratio increased. The presence of 0.15% α-tocopherol, or 0.5% CLnA, or the combination of 0.25% CLnA + 0.15% α-tocopherol in the diet, lowered total cholesterol and non-HDL-cholesterol levels in diabetic rats to similar extents. The combination of CLnA and α-tocopherol did not evoke responses that were significantly different from those evoked by α-tocopherol alone. Koba *et al.* (12) reported a significant lowering of serum cholesterol concentration in rats fed CLnA compared with those fed linoleic acid (LA). It was previously reported that α-tocopherol has cholesterol- and TAG-lowering effects (31). CLnA at a 0.5% level failed to reduce TAG levels significantly in the lipids of diabetic rats, as reported previously for nondiabetic rats (11). α-Tocopherol significantly lowered TAG levels in plasma of diabetic rats to levels found in nondiabetic control animals. The combination of CLnA (0.25%) with α-tocopherol also significantly reduced TAG levels, but these levels were not significantly different from α-tocopherol alone.

The effects of different synthetic and natural antioxidants as inhibitors of LDL peroxidation and their possible therapeutic effects to counteract atherogenesis are well documented (32,33). LDL-lipid peroxidation and its inhibition by antioxidants are important in the molecular mechanism of atherosclerosis. Superoxides generated in response to hyperglycemia lead to the peroxidation of lipids of plasma, lipoprotein, and EM (33). Oxidized lipoproteins lead to the development of atherosclerosis and diabetic vascular complications. In the present study, the CLnA-fed diabetic rats showed a significant decrease in EM LOS, which was equivalent to the α-tocopherol group. The plasma peroxidation showed no significant difference. The peroxidation level in DM was so high that neither 0.5% CLnA nor α-tocopherol, nor a combination of these agents, decreased peroxide production, although in normal rats the peroxidation level decreased with 0.5% CLnA intake (11).

It is interesting that CLnA, α-tocopherol, and their combination significantly decreased the EM lipid peroxidation level without changing the phospholipid and cholesterol content and their ratios. These data corroborate previous observations (11) that CLnA lowered EM lipid peroxidation in normal rats. It suggests that CLnA at a 0.5% level is effective in both normal and diabetic conditions as an antioxidant. However, the combination of 0.25% CLnA and α-tocopherol did not induce significantly different EM lipid peroxidation changes compared with animals fed α-tocopherol alone.

The liver total lipid, cholesterol, and phospholipid profiles showed no significant differences between the control and diabetic groups of rats irrespective of their dietary intake of CLnA and α-tocopherol or their combination. However, whereas CLnA alone and α-tocopherol alone failed to lower liver lipid peroxidation levels significantly in diabetic animals, liver lipid peroxidation was significantly reduced by about 50% in the group fed α-tocopherol together with a lower dose of CLnA.

A comparison of antioxidant activity between dietary CLnA and CLA has both importance and relevance in the context of claims made in favor of CLA. Kim *et al.* (34) reported that dietary CLA reduced lipid peroxidation by increasing oxidative stability in rats, increasing the proportions of higher saturated and monounsaturated FA in both plasma and hepatic membrane, while lowering levels of oxidatively susceptible PUFA such as linoleic, linolenic, and arachidonic acids. A similar reasoning may explain the relations of dietary CLnA and α-tocopherol in the lowering effect of lipid peroxidation.

A conservative explanation for the antioxidant activity of CLnA can be drawn based on the bioconversion reactions of CLnA. Under *in vivo* conditions, CLnA can undergo isomerization, hydrogenation, and oxidation reactions to form PUFA of both nonconjugated acids (LA and LnA) and conjugated CLA. It is known that CLA reduces superoxide dismutase activity while having no catalase-enhancing activity. This response may have enhanced the antioxidant activity of CLnA when used even at a reduced dose with the fixed dose of α-tocopherol.

The present report provides scope to investigate the effects of orally administered CLnA in diabetes (rats or human beings). The results of the present study demonstrate the beneficial effect of CLnA ameliorating the adverse free radical-generating influence of alloxan-induced DM. This is the first report demonstrating the synergistic effect of CLnA with α-tocopherol as *in vivo* antioxidative agents on lipid peroxidation in diabetic rats.

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Effect of Sucrose and Saturated-Fat Diets on mRNA Levels of Genes Limiting Muscle Fatty Acid and Glucose Supply in Rats

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ABSTRACT: In this study, we examined whether the increased availability of lipids in blood resulting from two types of diet manipulation regulated metabolic gene expression in the skeletal muscle of rats. Feeding for 4 wk on an isocaloric-sucrose or a hypercaloric-fat diet increased plasma TAG in the fed condition by increments of 70 and 40%, respectively, and increased fasting insulinemia (approximately 3-fold) compared with a starch diet. The fat diet impaired glucose tolerance and caused obesity, whereas sucrose-fed rats maintained their normal weight. We analyzed the expression of genes that regulate the exogenous FA supply (LPL, FAT/CD36, FATP1), synthesis (ACC1), glucose (GLUT4, GLUT1, HK2, GFAT1, glycogen phosphorylase) or glycerol (glycerol kinase) provision, or substrate choice for oxidation (PDK4) in gastrocnemius and soleus muscles at the end of the glucose tolerance test. LPL, FAT/CD36, FATP1, PDK4, and GLUT4 mRNA as well as glycogen phosphorylase and glycerol kinase activity levels in both muscles were unchanged by the diets. Increased mRNA levels of GLUT1 (1.6- and 2.6-fold, respectively) and GFAT1 (about 1.7-fold) in gastrocnemius, and of ACC1 (about 1.5-fold) in soleus, were found in both the sucrose and fat groups. In the fat group, HK2 mRNA was also higher (1.8-fold) in the gastrocnemius. Both sucrose and saturated-fat diets prompted hyperinsulinemia and hyperlipemia in rats. These metabolic disturbances did not alter the expression of LPL, FAT/CD36, FATP1, PDK4, and GLUT4 genes or glycogen phosphorylase and glycerol kinase activity levels in either analyzed muscle. Instead, they were linked to the coordinated upregulation in gastrocnemius of genes that govern glucose uptake and the hexosamine pathway, namely, GLUT1 and GFAT1, which might contribute to insulin resistance.

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In rats, hyperlipemia appears after a few weeks on diets that include sucrose (1) or fat (2) as the main nutrient. Nevertheless, sucrose (3–5) and fat (6–8) diets promote quite distinct metabolic phenotypes in rats. Isocaloric replacement of dietary starch by sucrose does not increase body weight (9–11), whereas the elevation of blood insulin is controversial, since normal (4,5) and high levels of insulin (12,13) have been reported. Hypercaloric fat diets, in turn, trigger obesity and insulin resistance (14). The mechanisms by which each type of diet raises blood lipids are distinct. In sucrose-fed rats, metabolic changes are mainly mediated by fructose, which induces hepatic lipogenesis (15). High-fat diets raise lipemia owing to the increased supply of FA (16).

Skeletal muscle is a key contributor to metabolite clearance. The competitive use of FA and glucose by the muscle tissue is considered a basic mechanism of normal metabolic homeostasis. However, there is scarce information (17,18) about the regulation, in hyperlipemic conditions, of genes that control the FA and glucose supply to skeletal muscle, which may contribute to this imbalance. A previous study analyzed the expression of FA transport and β-oxidation genes in the skeletal muscle of humans during a short-term (days) isocaloric fat diet (19). Key genes in muscle FA metabolism are: lipoprotein lipase (LPL), which hydrolyzes plasma lipoprotein TAG (20); FA translocase (FAT/CD36) (21,22) and the FABP FATP1 (23), which promote FA uptake; and the acetyl-CoA carboxylase ACC1 isoform, which is primarily involved in FA synthesis (24). Glycerol kinase may also be a relevant gene in FA metabolism, since it may limit FA esterification (25). Crucial genes involved in the regulation of muscle cell glucose disposal are the glucose transporters GLUT4 and GLUT1 (26); hexokinase, the predominant muscle isoform HK2, which catalyzes subsequent glucose phosphorylation (27); and glycogen phosphorylase, which controls the endogenous glucose supply from glycogen (28). Pyruvate dehydrogenase kinase, PDK4, which is primarily expressed in muscle, is a putative regulatory gene of glucose and FA selectivity for oxidation (29). PDK4 inactivates pyruvate dehydrogenase activity, the enzyme that limits the formation of acetyl-CoA from glucose, thereby negatively regulating glucose oxidation. Finally, the ubiquitous isoform of glutamine:fructose-6-phosphate amidotransferase, GFAT1, is the rate-limiting enzyme for hexosamine biosynthesis, which is a nutrient-sensing pathway that signals insulin resistance (30).

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Abbreviations: ACC1, acetyl-Co carboxylase 1; CT, threshold cycle; FAT/CD36, fatty acid translocase; FATP1, fatty acid transport protein 1; GFAT1, glutamine:fructose-6-phosphate amidotransferase 1; GLUT1 and GLUT4, glucose transporters; HK2, hexokinase 2; II_{30}, insulinogenic index after 30 min; LPL, lipoprotein lipase; PDK4, pyruvate dehydrogenase kinase, isoenzyme 4; QUICKI, quantitative insulin sensitivity check index; RPLP0, ribosomal protein large, P0; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine.

Thus, the aim of this study was to compare the effects of a sucrose-based diet vs. a starch-based diet on whole body insulin sensitivity and the expression of metabolic genes in skeletal muscle. An earlier study had compared the effects of a high-sucrose or high-fat diet on FA metabolic genes in the liver (31) exclusively. Here, mRNA levels of FA uptake- and deposition-regulating genes, such as LPL, FAT/CD36, FATP1, ACC1 or the glucose transporters GLUT1 and GLUT4; the glucose-metabolism-regulating genes GFAT1, HK2, and PDK4; and the enzyme activities of glycogen phosphorylase and glycerol kinase were assessed in soleus and gastrocnemius muscles.

EXPERIMENTAL PROCEDURES

Animals, diets, and food intake control. Male Wistar rats were purchased from Interfauna (Harlan Ibérica, Barcelona, Spain) and had an average weight of 200–225 g. They were placed in standard cages (two rats per cage) fitted with special powder feeders and kept at a constant temperature of 23°C with a 12-h light/dark cycle. Rats had free access to water and diet. During the first week of acclimation, they were fed a standard high-starch-based diet (control) (A04; Panlab, Barcelona, Spain). The composition was: 17.5% protein, 2.9% sucrose, 63.3% starch, 3.3% lard, 5.5% fiber, 1.0% vitamin mixture, 6.0% mineral mix, 0.3% DL-methionine, and 0.2% choline bitartrate (364 kcal/100 g). Thereafter, groups of 6 rats were fed the control diet or a sucrose- or lard-based diet for 4 wk. Sucrose and lard diets were prepared at the Nestlé Research Center (Lausanne, Switzerland). The composition of the two diets were: (i) sucrose [20% protein, 50% sucrose, 13.3% starch, 5% lard, 5% fiber, 1.5% vitamin mixture, 4.7% mineral mix, 0.3% DL-methionine, and 0.2% choline bitartrate (378 kcal/100 g)], and (ii) lard [25.4% protein, 8.5% sucrose, 16.9% starch, 33.9% lard, 8.4% fiber, 1.5% vitamin mixture, 4.7% mineral mix, 0.4% DL-methionine, and 0.3% choline bitartrate (508 kcal/100 g)]. Rats and food were weighed every 2 d to calculate weight gain and food intake, respectively. Animal treatment protocols were approved by the Comitè Etic d'Experimentació Animal of the University of Barcelona (Barcelona, Spain).

Glucose tolerance test and tissue sampling. Four weeks after dietary treatment, animals were fasted for 18 h and then anesthetized with pentobarbital (70 mg/kg of body weight), and an isotonic solution of glucose (2 g/kg of body weight) was administered by ip injection. Rats were always bled *via* the tail before and 10, 20, 30, 60, 90, and 120 min after glucose administration. At the end of the test (2 h after glucose injection), gastrocnemius and soleus muscles were excised and immediately frozen in liquid N_2 and stored at −80°C. The abdominal cavity was then opened and the epididymal fat pads were removed and weighed before being frozen in liquid N_2.

Measurement of metabolite and insulin. Plasma insulin levels were assayed by ELISA (Crystal Chem Inc., Chicago, IL). TAG and FFA were measured in plasma samples using kits from Sigma Chemical Co. (St. Louis, MO) and Roche (Basel, Switzerland). Lactate was measured enzymatically in plasma. Blood glucose was analyzed with a reflectometer (GlucoTouch®; Lifescan Johnson & Johnson, Milpitas, CA). QUICKI, a quantitative insulin sensitivity check index (32), was calculated as QUICKI = 1/[log(Gb) + log(Ib)], where Gb is fasting plasma glucose (mg/dL), and Ib is the fasting plasma insulin (μU/mL). The insulinogenic index at 30 min (II_{30}) (33) was calculated as the ratio of increase at 30 min of plasma insulin, $\Delta I_{30} = Ins_{30min}$ (mU/L) − Ins_{0min} (mU/L), vs. that of glucose, $\Delta Glu_{30} = glucose_{30min}$(mmol/L) − $glucose_{0min}$(mmol/L), $II_{30} = \Delta I_{30}/\Delta Glu_{30}$, during the glucose tolerance test.

Enzyme activity assays. Muscle samples were homogenized with a Polytron (Kynematica Polytron, Westbury, NY) in a buffer containing 50 mM Hepes (pH 7.8), 40 mM KCl, 11 mM $MgCl_2$, 1 mM EDTA, and 1 mM DTT to measure glycerol kinase, or in a buffer containing 10 mM Tris-HCl (pH 7.0), 150 mM KF, 15 mM EDTA, 600 mM sucrose, 15 mM 2-mercaptoethanol, 10 μg/mL leupeptin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride to measure glycogen phosphorylase. Homogenates were centrifuged at 10,500 × *g* for 15 min at 4°C, and the resulting supernatants were used. To quantify glycerol kinase activity, 5 μL of muscle supernatant (approximately 20–30 μg of protein) was mixed with 25 μL of reaction buffer containing 100 mM Tris (pH 7.4), 6 mM ATP, 13.3 mM $MgSO_4$, 100 mM KCl, 20 mM 2-mercaptoethanol, and 21 μM [U-^{14}C]glycerol (specific activity 5.25 GBq/mmol). Reactions were performed for 40 min at 37°C (progress curves were linear for more than 1 h in these conditions) and then terminated by addition of 50 μL of ice-cold 98% ethanol. A 30-μL quantity of reaction mixture was spotted onto DEAE-cellulose discs and then allowed to dry for 1 h at room temperature. The discs were placed on a filter holder attached to a vacuum tube and washed twice with 5 mL and once with 50 mL of water. After drying, the papers were dropped in vials with 4 mL of scintillation liquid and counted in a Wallac scintillation counter to quantify the [^{14}C]glycerol-3-phosphate retained. Glycogen phosphorylase activity was detected following the incorporation of [U-^{14}C]glucose 1-phosphate into glycogen in the absence or presence of the allosteric activator AMP (5 mM) (34). Protein concentration was measured (Bio-Rad, Hercules, CA).

RNA extraction, reverse transcription, and real-time PCR. Total RNA was extracted from muscle samples following the instructions of the RNeasy minikit (Qiagen, Valencia, CA). Extracts were homogenized with a Polytron (Kynematica Polytron). The integrity of isolated RNA was verified by electrophoresis and ethidium bromide staining. A 0.5-μg quantity of total RNA was retrotranscribed with TaqMan reverse transcription reagents from Applied Biosystems (Branchburg, NJ) using random hexamers. Real-time PCR was performed in the ABI PRISM 7700 sequence detection system with the TaqMan universal PCR master mix and probes for LPL, ACC1, GLUT1, GLUT4, FAT, FATP1, GFAT1, HK2, and PDK4 from Applied Biosystems. β2-microglobulin and ribosomal protein P0 (RPLP0) were used as endogenous controls to normalize the threshold cycle (CT) for each probe assay as stated. Real-time PCR was performed in duplicate. Relative gene expression was estimated as $2^{-\Delta CT}$ and gene fold change was estimated as $2^{-\Delta\Delta CT}$ (35).

TABLE 1
Body Weight Gain and Plasma Metabolites After Dietary Treatments in Fasted and Fed Animals[a]

	Control	Lard	Sucrose
Caloric intake (kcal/d)	51.1 ± 1.69	81.0 ± 16.2**	62.0 ± 4.4**
Body weight gain (g)	74.6 ± 6.8	104 ± 16.4*	68.2 ± 5.6
Epididymal WAT weight (g)	3.4 ± 0.4	8.1 ± 2.4**	5.3 ± 1.2*
Fed plasma TAG (mg/dL)	107 ± 9.17	150 ± 20.4**	186 ± 13***
Fasting plasma TAG (mg/dL)	87.5 ± 28.8	62.2 ± 28.6	57.5 ± 11.6
Fed plasma FFA (μM)	410 ± 40	728 ± 93***	507 ± 115
Fasting plasma FFA (μM)	830 ± 99	885 ± 126	870 ± 52
Fed plasma lactate (mg/dL)	42.2 ± 3.7	50.4 ± 4.7*	39.4 ± 1.1
Fasting plasma lactate (mg/dL)	19.3 ± 7.8	15.9 ± 8.4	16.0 ± 2.6

[a]Values are means ± SD of 6 subjects. The significance of the differences vs. controls was: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. WAT, white adipose tissue.

Statistical analysis. All data are presented as means ± SD and the significance of the difference was analyzed by Student's *t*-test, except real-time PCR data, which were examined using the Relative Expression Software Tool (36). Where significant differences were found, the *P* values have been stated in the figure legend. Values were considered significant at $P < 0.05$.

RESULTS

Body weight and plasma metabolites under diet treatments. Rats were fed a control, isocaloric sucrose-based, or hypercaloric lard-based diet *ad libitum* for 4 wk. Daily caloric intake was higher in animals fed on lard (60%) and sucrose (20%) than in controls (Table 1). At the end of the treatment, average body weight in the lard group was 40% higher than in controls, whereas no significant difference from controls was detected in the sucrose group. In contrast, the weight of epididymal adipose tissue was higher in the sucrose (1.5-fold) and lard (2.3-fold) groups than in controls.

Plasma TAG levels did not differ significantly between the three diet groups in 18 h-fasted animals (Table 1). Neither plasma FFA nor lactate were altered in this nutritional condition. In contrast, in the fed condition, TAG, FFA, and lactate were higher in the lard group by 40, 77, and 19%, respectively, than in controls. In the sucrose group, only the 70% rise in TAG was significant.

Plasma fasting glucose was the same in controls as in the sucrose group, whereas the lard group showed 15% higher levels (Table 2). Remarkably, clear-cut increments in fasting insulinemia were observed in the lard (3-fold) and sucrose (3.7-fold) groups compared with controls, suggesting insulin resistance. We used QUICKI as an index of insulin sensitivity in the fasting condition. This index highly correlates with the insulin response, as assessed by euglycemic hyperinsulinemic clamps (32). QUICKI was lower in the sucrose and lard groups compared with controls, indicating that the former two diets triggered insulin resistance. We then evaluated glucose tolerance. Two hours after intraperitoneal administration of a bolus of glucose, glycemia was 20% higher in the lard group only (Table 2). Glucose and insulin concentrations were measured 30 min after the glucose load and the insulinogenic index (II_{30}) was assessed. This index estimates the first phase of insulin response (33) and is based on the increase in insulinemia over 30 min (ΔI_{30}) relative to the increase in glucose concentration (ΔGlu_{30}). The ΔI_{30} was similar in controls and the sucrose group (mean values 143 and 149, respectively) but lower in the animals fed on lard (103). ΔGlu_{30} was comparably lower in animals fed a diet of lard or sucrose (mean values 4.6 and 4.5, respectively) than in controls (6.4). Therefore, the II_{30} (Table 2) was similar for the control and lard groups, whereas the sucrose group showed a 42% increase over controls.

mRNA levels of metabolic genes in soleus and gastrocnemius muscles. The relative mRNA levels of LPL, FAT/CD36,

TABLE 2
Glucose Tolerance and Insulin Sensitivity[a]

	Control	Lard	Sucrose
Plasma glucose, 0 min (mM)	4.26 ± 0.47	4.94 ± 0.48*	4.51 ± 0.41
Plasma glucose, 30 min (mM)	10.7 ± 3.50	9.55 ± 1.14	9.0 ± 1.36
Plasma glucose, 120 min (mM)	5.6 ± 0.52	7.2 ± 0.74**	6.9 ± 1.96
Plasma insulin, 0 min (mU/L)	16.4 ± 4.4	51.1 ± 28.4*	60.3 ± 28.1*
Plasma insulin, 30 min (mU/L)	160 ± 27.5	154 ± 40.0	210 ± 42.7
QUICKI	0.55 ± 0.04	0.43 ± 0.05**	0.41 ± 0.05**
Insulinogenic index (II_{30})	26.4 ± 11.0	25.3 ± 6.0	37.6 ± 10.9

[a]Blood glucose and plasma insulin were measured before (0 min), 30 min after, and 120 min after administration of glucose. Values are means ± SD of 6 subjects. The significance of the differences vs. time-matched controls was: *$P < 0.05$, **$P < 0.01$. QUICKI, a quantitative insulin sensitivity check index.

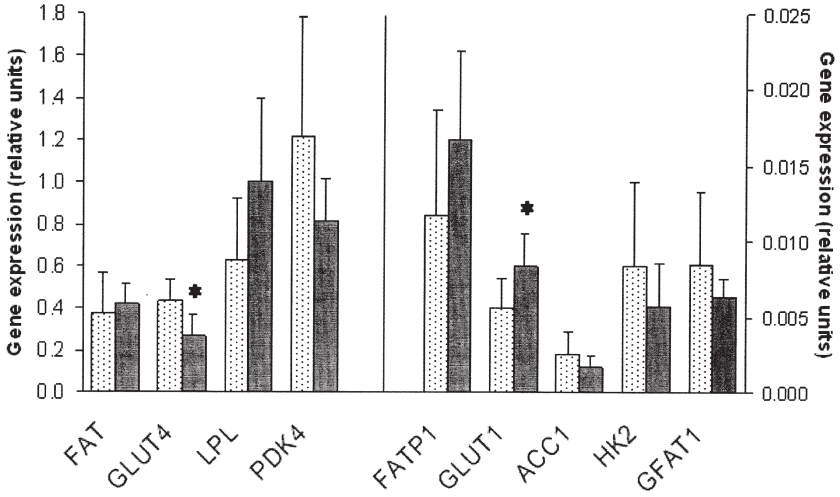

FIG. 1. Relative muscle gene expression. Animals were administered a control diet for 4 wk, fasted overnight, and subsequently subjected to a glucose tolerance test. Expression of LPL, GLUT1, GLUT4, FATP1, FAT, ACC1, GFAT1, HK2, and PDK4 was assessed by real-time PCR in soleus (dark bars) and gastrocnemius (dotted bars) muscles harvested at the end of the test. The threshold cycle (CT) values for the reference probe beta2-microglobulin were subtracted from FAT, FATP1, GLUT1, GLUT4, and LPL probes CT (ΔCT), whereas ACC1, GFAT1, HK2, and PDK4 were compared with RPLP0. Data are expressed as $2^{-\Delta CT}$. Values are means ± SD from 6 muscles of each type. The significance of the differences of gastrocnemius vs. soleus muscle was: $^*P < 0.05$.

FATP1, ACC1, GLUT4, GLUT1, GFAT1, HK2, and PDK4 were assessed in slow- twitch (soleus) and fast-twitch (gastrocnemius) muscles (37) by real-time PCR, using beta2-microglobulin and RPLP0 as endogenous controls. Expression of each control gene was the same in both muscles and all diet groups (data not shown). Significant differences were observed for (i) GLUT1, which was approximately 48% more abundant in soleus (Fig. 1), and for (ii) GLUT4, which was predominant in gastrocnemius (60% higher relative expression). In the sucrose and lard groups, the expression of the FABP FAT and FATP1 showed a tendency to decrease in gastrocnemius, but no clear trend was detected in soleus. However, these decreases were not significant (Table 3). ACC1 expression increased in the sucrose and lard groups in the soleus only (Table 3). In contrast, GFAT1 and HK2 showed a tendency to upregulate in the gastrocnemius only, although differences were not significant for HK2 in the sucrose diet (Table 3). No major effects were observed on LPL or PDK4 expression. Regarding the glucose transporters, GLUT4 expression was not significantly modified by diet treatments, whereas GLUT1 mRNA was upregulated exclusively in gastrocnemius in the sucrose and lard groups (Table 3).

TABLE 3
Fold Change in Muscle Gene Expression After Dietary Treatments[a]

	Soleus		Gastrocnemius	
	Sucrose	Lard	Sucrose	Lard
FAT	−1.25	−1.15	−1.30	−1.35
FATP1	−1.13	−1.05	−1.22	−1.32
GLUT1	−1.11	+1.03	+1.62*	+2.68**
GLUT4	−1.27	−1.23	−1.08	+1.00
LPL	−1.09	−1.11	+1.02	−1.40
ACC1	+1.52	+1.46**	+1.28	+1.24
GFAT1	+1.10	+1.26	+1.71*	+1.89*
HK2	−1.05	+1.09	+1.65	+1.88*
PDK4	−1.03	+1.23	−1.03	−1.01

[a]Expression of GLUT1, GLUT4, FATP1, FAT, LPL, ACC1, GFAT1, HK2, and PDK4 was assessed in muscles from 6 controls, 6 sucrose-fed animals, and 6 lard-fed animals subjected to a glucose tolerance test. The threshold cycle (CT) values for the reference probe beta2-microglobulin were subtracted from each probe as in Figure 1. Data are expressed as $2^{-\Delta\Delta CT}$ for upregulated genes and $1/2^{-\Delta\Delta CT}$ for downregulated ones. The former are shown by + and the latter by −. The significance of the differences was: $^*P < 0.05$ and $^{**}P < 0.01$.

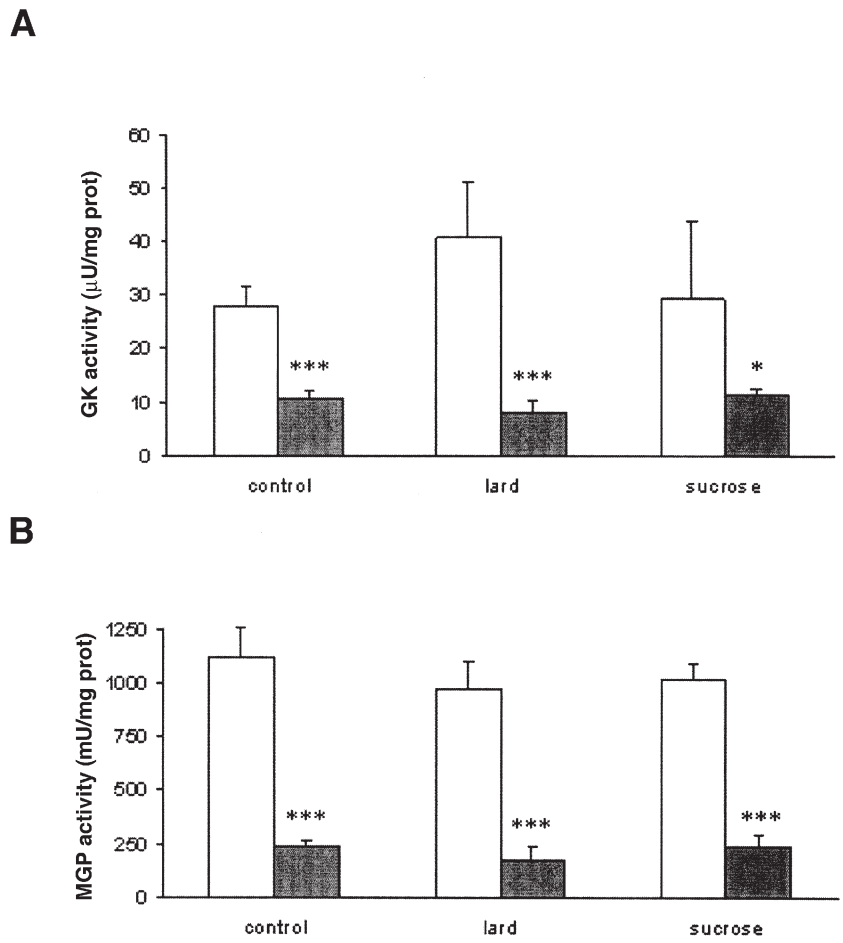

FIG. 2. Glycerol kinase and glycogen phosphorylase activities in muscle. Four weeks after dietary treatment, animals were fasted overnight and then subjected to a glucose tolerance test. At the end of the test, glycerol kinase (GK) (panel A) and glycogen phosphorylase (MGP) (panel B) activities were analyzed in extracts from soleus (dark bars) and gastrocnemius muscles (open bars). Values are means ± SD of 6 subjects. The significance of the differences of gastrocnemius vs. soleus was: $^{***}P < 0.001$ and $^{*}P < 0.05$.

Activity of glycerol kinase and glycogen phosphorylase in the muscle tissues. In controls, glycogen phosphorylase and glycerol kinase activities were higher in gastrocnemius than in soleus, by 4.6- and 2.7-fold, respectively. The administration of the two experimental diets did not alter these enzyme activity levels in either muscle type (Fig. 2).

DISCUSSION

This study shows that the sucrose-based diet, like the lard diet, impaired insulin sensitivity, as assessed by the increase in fasting plasma insulin and the reduction in the insulin sensitivity index, the QUICKI value. This is a reliable, easily applicable index used to estimate insulin effectiveness in humans (32) and is suitable for rats (6) and mice (38) as well, based on the increase in fasting insulin and glucose concentrations. Previous data on fasting insulin levels after sucrose nurturing are inconsistent. Certain authors have reported no increase 4–5 wk after administration of sucrose to rats, although the euglycemic–hyperinsulinemic clamp technique revealed insulin resistance (4,5), whereas others have described slight (12) or even marked fasting hyperinsulinemia (13). On the other hand, fat diets have consistently been shown to induce hyperinsulinemia (6,14) and to lower insulin sensitivity index (QUICKI) values (6).

The lard diet significantly impaired glucose tolerance, as assessed by the higher 120-min glycemia after administration of a glucose bolus, whereas the sucrose diet did not. Nevertheless, glucose clearance in hyperglycemic–hyperinsulinemic conditions, during the first 30 min after the glucose bolus, was enhanced by both diets, as deduced from the lower 30-min glucose increase (ΔGlu_{30}). On the other hand, the 30-min insulin increase (ΔI_{30}), which is a measure of insulin secretion (39), was steady in the sucrose group but lower in the lard group. Thus, the insulinogenic index (II_{30}), which correlates well with early-phase insulin response to the intravenous glucose tolerance test in humans (39), was higher in the sucrose group but remained unchanged in the lard group.

Both sucrose and fat diets increased postprandial plasma TAG and FA. Since little is known about potential alterations in gene expression in skeletal muscle, which may contribute to this metabolic disturbance, we performed a comparative analysis in soleus and gastrocnemius muscles at the end of the glucose tolerance test in rats fed starch-, sucrose-, and saturated fat-based diets. The uptake of FA in muscle may have been restricted by the activity of LPL, which hydrolyzes plasma lipoprotein TAG, and by the cell content of FABP, such as FAT/CD36 and FATP1, which respectively facilitate the transport and import of these molecules (21–23). However, we found that the mRNA of LPL, FAT, and FATP1 in rats fed on sucrose or lard did not differ significantly from those of controls. Of note, the FATP1 protein content was lower in the gastrocnemius of animals fed lard than in those fed starch (6). We also examined the expression of acetyl-CoA carboxylase ACC1, a cytosolic protein that catalyzes the formation of malonyl-CoA for the *de novo* synthesis of FA (24). ACC1 mRNA levels increased significantly in soleus muscle. The conditions that increased the expression of ACC1 in liver (40,41) were associated with the marked enhancement of lipid output and thus hyperlipemia (40), but there is no information on the regulation of ACC1 expression or the consequences of its higher expression in muscle.

Next, we tested whether the genes related to glucose clearance were altered. Glucose uptake was restricted by the glucose transporters, mainly GLUT4 and GLUT1 (26). Thus, we examined whether the expression of these genes was affected. The expression of the GLUT4 isoform was not altered by either experimental diet. However, the translocation of this transporter may have been impaired since the response of fast- and slow-twitch skeletal muscle glucose transport to stimulation by insulin or contraction decreased in rats fed a sucrose (3,5) or fat diet (17,18). In contrast, GLUT1 gene expression in gastrocnemius muscle was slightly increased by the sucrose diet and was more than doubled by lard. To our knowledge, there is only one report of GLUT1 upregulation associated with insulin desensitization, performed in patients with acanthosis nigricans, in which GLUT4 expression was also normal (42). High expression of GLUT1 in the muscle of transgenic mice enhances glucose uptake at high glucose concentrations but impairs the response to insulin (43). Thus, the increase in GLUT1 in the muscle of animals fed on sucrose or lard may be related to the two conditions observed: improved glucose clearance in hyperglycemic conditions and insulin resistance. Glucose uptake is also limited by hexokinase activity. We found that the lard diet increased the expression of the main muscle isoform, HK2, exclusively in gastrocnemius. The sucrose diet showed the same tendency, although differences did not reach significance. Our data are consistent with a previous finding that the HK2 gene is stimulated by insulin in either the cultured muscle cell line L6 (44) or in rat muscle *in vivo* after use of a hyperinsulinemic clamp method (27). Finally, we found that expression of the ubiquitous isoform of GFAT1 in muscle was increased by both sucrose and lard diets, again exclusively in gastrocnemius. This enzyme limits the hexosamine synthethic pathway, whose terminal metabolite is uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) (30), a donor substrate for the addition of GlcNAc to the hydroxyl groups of serine or threonine residues of proteins (45). Augmentation of this pathway enhanced O-GlcNAc modification of cell proteins (45) and generated insulin resistance, as shown by overexpression of the gene in muscle and adipose tissues (30). These findings therefore suggest that increases in GFAT1 expression in the muscle of hyperlipemic animals fed sucrose and lard diets may contribute to insulin resistance.

We also tested whether glycogen phosphorylase, which limits glycogen hydrolysis, was regulated through the diet. Animals fed a lard- or sucrose-based diet showed no significant alteration compared with controls. Glycerol kinase levels were also assessed, since glycerol 3-phosphate, which may limit FA esterification and disposal, may be generated by direct uptake of glycerol and subsequent phosphorylation by this enzyme (46). Glycerol kinase activity was not affected by either experimental diet. Finally, we tested whether the expression of PDK4 was affected. PDK4 expression in muscle has been shown to be upregulated by starvation and streptozotocin-induced diabetes in rats (47). However, we found no changes resulting from either diet, providing further evidence that, under diet-induced hyperinsulinemic conditions, gene expression is not suppressed.

In conclusion, we showed that the sucrose diet caused hyperinsulinemia and hyperlipemia in rats, as did the saturated-fat diet. Neither of these diets altered the expression in muscle of a number of genes involved in FA or glucose uptake, namely, LPL, the FABP FAT/CD36 or FATP1 and GLUT4, or of the enzymes responsible for the endogenous supply of glucose or glycerol, such as glycogen phosphorylase and glycerol kinase, or of PDK4, the regulator of glucose oxidation. In contrast, both diets regulated the mRNA levels of a number of metabolic genes in muscle in a similar fashion, although the effects of the sucrose diet were less pronounced. mRNA levels may be regulated by stability changes as well as transcriptional activity in response to the changes in diet. On the other hand, although changes were noted in certain gene mRNA levels, they may not directly correlate with translated protein levels or by enzyme activities that were not determined in these tissues. Genes involved in glucose uptake, GLUT1 and HK2, as well as diversion toward hexosamine synthesis, GFAT1, were upregulated in gastrocnemius muscle, whereas the FA synthetic gene ACC1 was induced in soleus muscle. Nevertheless, overexpression of both GLUT1 and GFAT1 has been associated with impaired insulin response, which suggests that this coordinated upregulation may contribute to sucrose- and high-fat diet-induced insulin resistance.

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Lymphatic Transport of a Physical Mixture of Medium- and Long-Chain TAG in Rats

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ABSTRACT: Lymphatic transport of a mixture of medium-chain TAG (MCT) and long-chain TAG (LCT) was studied in lymph-cannulated rats. Animals were administered a test emulsion containing either triolein, tricaprylin, or a 1:1 mixture of triolein and tricaprylin, and the lymph was collected for 24 h. The lymphatic recovery rate of medium-chain FA (MCFA) was significantly higher in rats given the TAG mixture than in those given MCT alone. The lymphatic recovery rate of long-chain FA (LCFA) also was significantly higher in rats given the TAG mixture than in those given LCT alone. No TAG containing three MCFA (i.e., MCT) was detected, and 37.7% of TAG containing one or two MCFA was detected in the lymph TAG when rats were given the TAG mixture. These results indicate that lymphatic transport of MCFA and LCFA can be modified by the combination of MCT and LCT.

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Medium-chain TAG (MCT) has been used as the fat source for enteral nutrition because of its absorption characteristics (1). When an enteral nutrient is compounded of MCT, long-chain TAG (LCT) is also added to supply EFA (2). However, dietary MCT is metabolized differently from LCT. Ingested LCT is degraded to FA and 2-MAG by pancreatic lipase in the upper intestine. In intestinal mucosal cells, the 2-MAG is reesterified with FA of exogenous or endogenous origin (3). Resynthesized TAG in the intestinal mucosa forms chylomicrons and flows into the venous system *via* lymphatic vessels. In contrast, ingested MCT is more easily and completely degraded to FA and glycerol than is LCT (4). Long-chain FA (LCFA) are activated to their CoA derivatives through the action of fatty acyl-CoA ligase and are resynthesized to TAG together with 2-MAG by acyltransferase. The ligase has low activity toward medium-chain FA (MCFA) (5). Therefore, most MCFA absorbed by the small intestinal mucosa are bound to albumin without the resynthesis of TAG and are transferred to the liver *via* the portal vein (6). Ikeda *et al*. (7) reported that the lymphatic absorption rate of caprylic acid was only 4% in rats given tricaprylin, whereas the rate of linoleic acid was 77% in those given trilinolein.

The lymphatic absorption rate of MCFA is affected by giving TAG simultaneously. Lee *et al.* (8) reported that when MCT was given with LCT, there was at most a threefold increase in the quantity of MCFA transported as TAG in the lymph. However, no information is available about the type of TAG in the lymph. Reesterification of MCFA may be promoted by the simultaneous intake of LCT. To determine the mechanism responsible for the enhanced MCFA transport by LCT, we analyzed the composition and regiospecificity of the lymph TAG in lymph-cannulated rats given a physical mixture of MCT and LCT.

MATERIALS AND METHODS

This study was carried out under the approval of the Laboratory Animal Care Committee of the Research Laboratory, Nisshin Oillio Group. Male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) weighing 250–280 g were used. The surgery and lymph collection were completed according to the method of Ikeda *et al.* (9). Briefly, under Nembutal anesthesia, animals were subjected to cannulation of the left thoracic lymphatic channel, and an indwelling catheter was placed in the stomach. The next morning, after the 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. The test emulsion contained 200 mg of sodium taurocholate, 50 mg of FA-free albumin, and 200 mg of either LCT (triolein), MCT (tricaprylin), or a 1:1 mixture of MCT and LCT. Triolein (purity 99%) and tricaprylin (purity 99%) were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Lipids of the lymph collected for 0–24 h after administration of the test emulsion were extracted according to the method of Folch *et al.* (10). The FA composition of the lymph total lipids was determined by GLC as described earlier (11), using heptadecanoic acid (Sigma-Aldrich, St. Louis, MO) as an internal standard. The FA recovery rate was calculated by subtracting the amount of FA in the blank lymph from that in the lymph collected after administration of the test emulsion. After trimethylsilylation, the TAG composition in the lymph collected for 0–4 h after administration of the emulsion was analyzed by GLC with a capillary column (DB-1ht; Agilent, Palo Alto, CA). Trimethylsilylation was performed using a trimethylsilylating

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Abbreviations: 1M2L, TAG containing one MCFA and two LCFA; 2M1L, TAG containing two MCFA and one LCFA; 3L, TAG containing three LCFA; MCFA, medium-chain FA; MCT, medium-chain TAG; LCFA, long-chain FA; LCT, long-chain TAG.

reagent (TMS-HT; TCI, Tokyo, Japan) according to the manufacturer's instructions. The reagent was a pyridine solution containing hexamethyldisilazane and chlorotrimethylsilane. Approximately 1 mg of TAG and 0.2 mL of the trimethylsilylating reagent were mixed, and the mixture was left at room temperature for 30 min. One milliliter of hexane and distilled water was added for phase separation. After centrifugation, the organic phase was subjected to GLC. Regiospecific analysis of the lymph TAG collected for 0–4 h after administration of the test emulsion was performed by silver-ion HPLC as described by Adlof (12). A Toso 8020 series HPLC system (Toso, Tokyo) with a column containing silver ions (ChromSpher 5 Lipids; Varian, Palo Alto, CA) was used. Lymph lipids (10 mg) were dissolved in 1 mL hexane and filtered through a polytetrafluoroethylene filter before injecting 3 μL of lipids into the HPLC instrument. HPLC-grade hexane with 0.5% acetonitrile was used in the mobile phase at a flow rate of 1.0 mL/min. Analyses of lymph TAG, FFA, phospholipids, and cholesterol were carried out on a model 7450 automated enzymatic system (Hitachi, Tokyo, Japan).

All statistical analyses were performed with SPSS for Windows, version 10.0 J (SPSS Japan, Inc., Tokyo, Japan). Data were expressed as the mean ± SEM. Data on the lymph flow and recovery of FA were analyzed by two-way ANOVA. Statistical differences in lipid levels in the lymph were determined by one-way ANOVA, and significance between the different groups was tested by the Tukey test. The result of the regiospecific analysis was determined by Student's unpaired *t*-test. All statistical tests were considered significant at $P < 0.05$.

RESULTS

The differences among TAG contained in the test emulsion did not affect the lymph flow rate (Fig. 1A). The lymphatic recovery rate of caprylic acid was significantly higher in the mixture group than in the MCT group (Fig. 1B). The lymphatic recovery rate of oleic acid was slightly but significantly higher in the mixture group than in the LCT group (Fig. 1C).

As shown in Table 1, there were no significant differences in the FFA and cholesterol concentrations of the lymph collected for 0–4 h. The concentration of lymph phospholipids was significantly lower in the MCT group than in the LCT and mixture groups. The TAG concentration in the lymph was highest in the LCT group and lowest in the MCT group, with the mixture group being in-between; the difference among groups was significant.

The TAG composition and concentration of lymph collected for 0–4 h are shown in Table 2. Only a negligible level (0.1 ± 0.1%) of MCT was detected in lymph TAG when rats were given MCT. No MCT was detected in the LCT group or in the mixture group. The proportions of TAG containing two MCFA and one LCFA (2M1L) were the same in rats given MCT and those given a mixture of MCT and LCT, whereas those of TAG containing one MCFA and two LCFA (1M2L) were significantly higher when the mixture was given than when MCT alone was given: 17.8 ± 1.5% and 31.0 ± 0.8%, respectively.

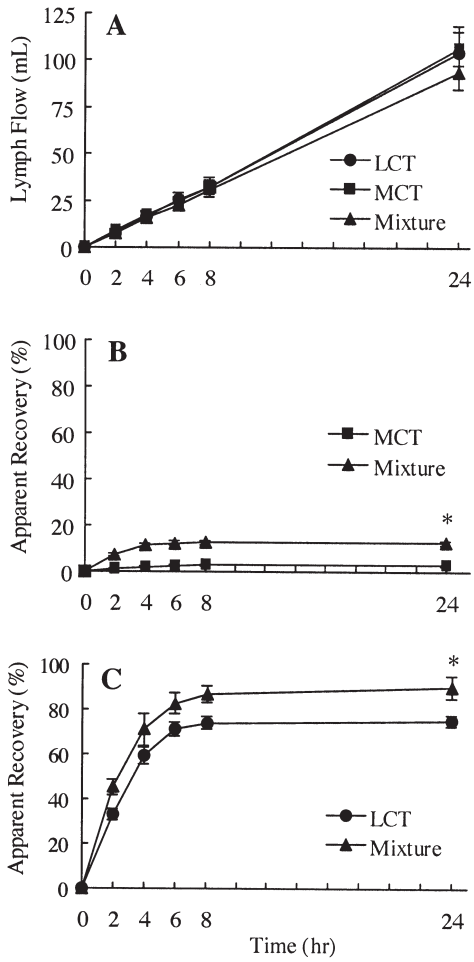

FIG. 1. Lymph flow (A) and recovery of caprylic acid (B) and oleic acid (C) in the thoracic duct lymph of rats after intragastricadministration of long-chain TAG (LCT), medium-chain TAG (MCT), or a mixture of MCT and LCT. Data are means ± SEM, $n = 4$ to 6. Asterisks indicate significant difference ($P < 0.05$).

Concentrations of 2M1L and 1M2L were significantly higher in the mixture group than in the MCT group, whereas those of TAG containing three LCFA (3L) were significantly higher in the LCT group than in the MCT and mixture groups. The 3L level of the LCT group was more than 10 times that of the MCT group, and was approximately twice that of the mixture group.

FA compositions of lymph collected for 0–4 h are shown in Table 3. The proportion of oleic acid was significantly higher when LCT alone was given than when the mixture was given: 80.4 ± 1.9% and 60.8 ± 1.8%, respectively. The proportion of caprylic acid in the MCT group was only 8.3 ± 1.2% when MCT alone was given. The proportions of palmitic and linoleic acids in the MCT group were 27.3 ± 2.0% and 24.7 ± 0.5%, respectively.

Results of the regiospecific analysis of the lymph TAG collected for 0–4 h in the mixture group are shown in Table 4. The proportion of symmetrical TAG was significantly lower, whereas that of asymmetrical TAG was significantly higher in the 2M1L-type TAG than in the 1M2L-type TAG.

TABLE 1
Concentration of Lymph Lipids Collected for 0–4 h in Rats Given Long-Chain TAG (LCT), Medium-Chain TAG (MCT), or a Mixture of MCT and LCT[a]

Lipids	Groups		
	LCT	MCT	Mixture
	(mmol/L)		
TAG	13.33 ± 1.60[c]	1.52 ± 0.27[a]	8.03 ± 0.39[b]
FFA	1.54 ± 0.55	1.12 ± 0.64	0.55 ± 0.06
Phospholipids	1.17 ± 0.12[b]	0.45 ± 0.05[a]	1.08 ± 0.04[b]
Cholesterol	0.43 ± 0.05	0.34 ± 0.10	0.56 ± 0.04

[a]Data are means ± SEM, $n = 4$ to 6. Values with different roman superscripts are significantly different ($P < 0.05$).

DISCUSSION

We studied the effects of LCT on the transport of MCFA and the TAG composition in the lymph of rats using MCT, LCT, or a mixture of MCT and LCT. The lymphatic recovery rate of caprylic acid was four times higher in the mixture group than in the MCT group. We thus confirmed the observation of Lee *et al.* (8) that in rats, the lymphatic transport of MCFA is enhanced when it is administrated simultaneously with LCT. No MCT was detected in lymph TAG, even when rats were given MCT as the sole source of TAG, and most of the MCFA occurred as TAG containing both MCFA and LCFA. Nor was MCT detected in the lymph TAG of rats given the mixture, and a considerable proportion was detected as TAG of the MCFA–LCFA type. These results indicate that a portion of the MCFA in the MCT administered, after partial hydrolysis, could have been reesterified with endogenous or exogenous LCFA in the enterocytes. Christophe *et al.* (13) reported that after feeding LCT and MCT to patients with ascitic fluid accumulation, the ascitic fluid contained TAG consisting of both MCFA and LCFA, indicating that part of the MCFA was absorbed as TAG containing LCFA.

TABLE 2
TAG Composition and Concentration of Lymph Collected for 0–4 h in Rats Given LCT, MCT, or a Mixture of MCT and LCT[a]

	Groups		
	LCT	MCT	Mixture
Composition (% of total TAG)			
3M	ND	0.1 ± 0.1	ND
2M1L	ND	6.8 ± 1.0	6.7 ± 0.4
1M2L	ND	17.8 ± 1.5[a]	31.0 ± 0.8[b]
3L	100.0 ± 0.0[c]	70.4 ± 2.4[b]	62.3 ± 1.2[a]
Concentration (mg/mL)			
3M	ND	0.00 ± 0.00	ND
2M1L	ND	0.10 ± 0.03[a]	0.46 ± 0.04[b]
1M2L	ND	0.25 ± 0.06[a]	2.14 ± 0.14[b]
3L	11.76 ± 1.4[b]	0.93 ± 0.15[a]	4.27 ± 0.20[a]

[a]Data are means ± SEM, $n = 4$ to 6. M, medium-chain FA; ND, not detected; L, long-chain FA; for other abbreviations see Table 1.

TABLE 3
FA Composition of Lymph Collected for 0–4 h in Rats Given LCT, MCT, or a Mixture of MCT and LCT[a]

FA	Groups		
	LCT	MCT	Mixture
	(% of total FA)		
8:0	ND	8.3 ± 1.2	6.3 ± 0.4
16:0	6.3 ± 0.7[a]	27.3 ± 2.0[c]	10.3 ± 0.4[b]
16:1	ND	0.6 ± 0.1[b]	0.3 ± 0.0[a]
18:0	3.0 ± 0.4[a]	11.5 ± 0.6[c]	4.7 ± 0.2[b]
18:1	80.4 ± 1.9[c]	15.6 ± 3.9[a]	60.8 ± 1.8[b]
18:2	6.7 ± 0.6[a]	24.7 ± 0.5[c]	9.9 ± 0.4[b]
18:3	0.4 ± 0.2	0.6 ± 0.2	0.2 ± 0.0
20:1	0.2 ± 0.1[a]	1.6 ± 1.0[a,b]	2.2 ± 0.5[b]
20:4	3.1 ± 0.4[a]	9.7 ± 0.6[c]	5.3 ± 0.4[b]

[a]Data are means ± SEM, $n = 4$ to 6. For abbreviations see Tables 1 and 2.

The lymphatic recovery rate of oleic acid was 15% higher in the mixture group than in the LCT group, indicating that MCT could promote lymphatic recovery of LCT when the two are given together. Christensen *et al.* (14) observed that lymphatic absorption of LCFA (linoleic and linolenic acid) was higher in rats given a mixture of MCT and soybean oil than in those given soybean oil alone. In contrast, Straarup and Høy (3) failed to find evidence of a promoting effect of MCT on lymphatic absorption of LCFA as rapeseed oil (oleic and linoleic acids). Christensen *et al.* (14) used MCT containing mainly caprylic acid, and Straarup and Høy (3) used MCT containing only capric acid. The type of MCFA may influence the LCFA absorption, as Ikeda *et al.* (7) reported the lymphatic absorption rate of caprylic acid to be lower than that of capric acid in rats.

In this study, we did not monitor the portal vein transport of MCFA and LCFA. Therefore, the total absorption rate (portal vein and lymph) of these FA was not clear. The lymphatic recovery rate of caprylic acid was significantly higher when rats were given the mixture of MCT and LCT than when they were given MCT alone. Most MCFA is absorbed *via* the portal vein. It is a reasonable inference that a portion of the MCFA principally absorbed *via* the portal vein was transported to the lymph. The lymphatic recovery rate of oleic acid was significantly higher when rats were given the mixture of MCT and LCT than when they were given the LCT alone. Because most LCFA is transported to the lymph vessels, the enhanced lymphatic recovery rate of oleic acid in the mixture group may indicate an increase in the total absorption of oleic acid.

During the intestinal absorption of the mixture of LCT and MCT, TAG with LCFA in the *sn*-2 position and MCFA in the *sn*-1,3 position (M-L-M) or TAG with LCFA in the *sn*-2 position and MCFA and LCFA in the *sn*-1,3 position (M-L-L or L-L-M) could be synthesized from long-chain 2-MAG and MCFA in the enterocytes. Also, TAG with MCFA in the *sn*-2 position and LCFA in the *sn*-1,3 position (L-M-L) or MCFA and LCFA in the *sn*-1,3 position (M-M-L or L-M-M) could be synthesized from LCFA and the partial hydrolysis products of MCT. All these types of TAG were detected in the lymph of rats given the TAG mixture. This result thus indicates that TAG

TABLE 4
Result of Regiospecific Analysis of Lymph TAG Collected for 0–4 h in Rats Given a Mixture of MCT and LCT[a]

	2M1L (% of total 2M1L TAG)	1M2L (% of total 1M2L TAG)
Symmetric	18.0 ± 2.3 (M-L-M)	33.8 ± 2.0* (L-M-L)
Asymmetric	82.0 ± 2.3 (M-M-L or L-M-M)	66.2 ± 2.0* (M-L-L or L-L-M)

[a]Data are means ± SEM, $n = 4$ to 6. Asterisks indicate significantly different from 2M1L ($P < 0.05$). For abbreviations see Tables 1 and 2.

containing MCFA and LCFA could be resynthesized from LCT and MCT backbones in the intestinal mucosa.

When rats were given only MCT, 27% of palmitic acid and 25% of linoleic acid were detected in the lymph FA. In contrast, only 8% of caprylic acid was detected. Only 0.1% of MCT was detected in lymph TAG after the administration of MCT alone. Most of the MCFA was recovered in the lymph as TAG composed of both MCFA and LCFA. These results indicate that MCFA is transported to the lymph after it is resynthesized with endogenous LCFA when rats are given MCT alone.

The TAG concentration of the lymph was correlated with the lymphatic recovery rate of caprylic and oleic acids, respectively. The concentration of lymph phospholipids in the MCT group was lower than those of the LCT and mixture groups. The secretion of phospholipids into the lymph increases during chylomicron secretion, because they are constituents of the chylomicrons. The lower concentration of lymph phospholipids may be related to a decrease in chylomicron secretion of the MCT group compared with the LCT and mixture groups.

In conclusion, the results of the present study indicate that the enhanced lymphatic transport of MCFA by a simultaneous administration of LCT is related to the synthesis of TAG containing MCFA and LCFA in the intestinal mucosa. This study also demonstrates that MCT enhances the transport of LCT. Thus, the lymphatic transport of MCFA and LCFA can be modified by the combination of MCT and LCT. This observation, together with the enhancement of LCT absorption by MCT, may contribute to the utilization not only of the physical mixture but also of the structural mixture (3,7,14,15) of MCT and LCT in various aspects of lipid nutrition.

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Monitoring Monohydroperoxides in Docosahexaenoic Acid Using High-Performance Liquid Chromatography

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ABSTRACT: The oxidation of free DHA has been investigated with respect to monohydroperoxides and polyhydroperoxides, which were analyzed with a novel HPLC method. The temperature and physical system, i.e., bulk and liposome, were varied. We have also studied the effects of antioxidants such as α-tocopherol, ascorbic acid, and juice from sea buckthorn on DHA. The HPLC method, which was performed isocratically, eluted eight peaks, each containing one or two isomers of monohydroperoxy-DHA. This method showed that the double bond farthest from the carboxyl group was easily oxidized, as shown by the rapid increase in the amount of C20-monohydroperoxy-DHA, which always provided the largest contribution to the total amount of monohydroperoxides. The monohydroperoxy-DHA containing the hydroperoxy group located on the double bond nearest the carboxyl group also was shown to increase considerably during an increase in the total amount of monohydroperoxides. This demonstrates that the double bonds located nearest and farthest from the carboxyl group were the most prone to hydroperoxide formation. DHA was more stable when stored in liposomes than as bulk. Addition of α-tocopherol to the DHA-containing liposomes reduced the oxidation of these double bonds. The antioxidant effect of α-tocopherol was prolonged when combined with ascorbic acid, since α-tocopherol was regenerated.

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Auto-oxidation of PUFA such as the n-3 FA DHA produces primary oxidation products, which may be further oxidized to secondary oxidation products. The oxidizability increases with the number of bis-allylic positions within a FA, and thus DHA with five bis-allylic positions is highly prone to oxidation (1). The oxidation of this PUFA is usually initiated by the loss of one hydrogen atom to another radical (1). The FA radical formed can react with oxygen and then take a hydrogen atom from another PUFA. The hydroperoxy FA produced is regarded as a primary oxidation product, which can be further oxidized by, for example, cyclization into hydroperoxy epidioxides and bicycloendoperoxides, which may be broken down into malonaldehyde. Another oxidation path of the hydroperoxy FA is linkage to another PUFA to form a dimeric compound. A third oxidation path is the cleavage of the FA containing the hydroperoxide.

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Abbreviation: Q-TOF, quadrupole time-of-flight.

Addition of antioxidants is a common method of protecting PUFA. Some antioxidants are classified as chain-breaking or primary antioxidants, which react with the FA radical or peroxyl or alkoxyl radicals and can thus inhibit or reduce oxidation (2). Other antioxidants are classified as preventive or secondary antioxidants, which reduce the oxidation rate (2). Within this class, the metal inactivators are considered to be the most important because they deactivate metal ions, which induce both initiation and decomposition of hydroperoxides (1). Combining a chain-breaking (such as tocopherol) and preventive (such as citric acid) antioxidant usually leads to significant synergistic effects.

To evaluate strategies for protecting PUFA such as DHA, suitable methods are needed to analyze the oxidation products. The most suitable method depends on the oxidation conditions. Under mild and harsh oxidation conditions, primary and secondary oxidation products dominate, respectively. Two methods of determining the total amount of primary oxidation products are measurements of the peroxide value or the conjugated dienes (1). In some situations, however, it is necessary to obtain information on where the hydroperoxide groups are situated in the DHA molecule. In such cases, the isomers must be separated, either with GC or HPLC. The drawback of GC is the need for derivatization of hydroperoxy-DHA before analysis (3). Since the hydroperoxy-DHA can be analyzed as is with HPLC (4), this type of analysis is preferable.

The aim of this study was to develop a new HPLC method for investigating the stability of DHA when both temperature and the type of physical system were varied. This method was also used to monitor the oxidation of DHA liposomes containing antioxidants when incubated at 40°C. The method was used to quantify several isomer groups of monohydroperoxy-DHA and the total amount of polyhydroperoxides. The isomers eluted using HPLC were characterized with quadrupole time-of-flight (Q-TOF) tandem MS. The advantage of this HPLC method is that the isomers are eluted isocratically. This was achieved with a shorter analysis time than when using a previously described thermospray LC-MS method (4) using a gradient.

MATERIAL AND METHODS

Materials. DHA (95% purity) was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Acetonitrile (HPLC grade) and acetic acid (analytical grade) were bought from

Merck (Darmstadt, Germany). L-α-PC (L-α-lecithin consisting of about 40% L-α-PC), L-ascorbic acid (vitamin C), and α-tocopherol (vitamin E) were purchased from Sigma (St Louis, MO). $FeSO_4 \cdot 7H_2O$ was purchased from the Aldrich Chemical Company (Milwaukee, WI). Sea buckthorn berries (*Hippophae rhamnoides*) were donated by SLU (Balsgård, Sweden) and processed at 80°C by the Department of Food Technology at Lund University (Lund, Sweden). These berries are rich in vitamin C.

Analysis of mono- and polyhydroperoxy-DHA. HPLC analysis of different monohydroperoxides and the total amount of polyhydroperoxides in DHA was carried out on a LaChrom system (Hitachi High Technologies America, Schaumburg, IL) consisting of a D-7000 interface, an L-7100 pump, an L-7250 autosampler (20 µL injection loop) and an L-7400 UV detector (offset at 234 nm). The separation of different monohydroperoxides in DHA was performed on a Lichrospher® 100 RP-18 (5 µm) column (Merck, Darmstadt, Germany) with a 1 mL/min flow rate of mobile phase consisting of an acetonitrile/aqueous solution of acetic acid (1%) 55:45 (vol/vol) for 40 min. The monohydroperoxy-DHA eluted within 19–36 min, while the polyhydroperoxides eluted between 1 and 10 min. The total amount of polyhydroperoxides was obtained from the sum of the peaks between 1 and 10 min. To prevent DHA accumulating in the column, each analysis was terminated with an increase in the acetonitrile concentration to 90% for 1 min, after which the concentration was maintained for 9 min. The acetonitrile concentration was then decreased to 55% for 1 min to equilibrate the column before the next analysis. This concentration was maintained for 14 min.

To be able to quantify the different isomers, the HPLC equipment was calibrated against a spectrophotometer. The model substance was *p*-nitrophenol dissolved in a mobile phase consisting of 0.2 M sodium acetate buffer pH 4.5/methanol 50:50. Several concentrations of *p*-nitrophenol were analyzed on a UV-1650 PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and on the HPLC equipment described above. Detection in both measurements was performed at a wavelength of 305 nm. By assuming that all monohydroperoxy-DHA isomers have an extinction coefficient (ε) of 23,300 $(M \cdot cm)^{-1}$ (5), it was possible to calculate the concentration with Equation 1. The extinction coefficient of *p*-nitrophenol, $\varepsilon_{p\text{-nitrophenol}}$, was obtained from the measurements on the spectrophotometer, whereas the calibration slope of *p*-nitrophenol ($HPLC_{p\text{-nitrophenol}}$) was obtained from the HPLC measurements.

$$\text{concentration} = \frac{\varepsilon_{p\text{-nitrophenol}}}{HPLC_{p\text{-nitrophenol}} \cdot \varepsilon_{\text{conjugated-diene}}} \cdot \text{area} = k \cdot \text{area} \quad [1]$$

The value of k was determined to be $4.029 \cdot 10^{-11}$ M/area unit.

Analysis of DHA content. To determine the DHA content, analysis was performed with a light-scattering detector added to the same HPLC system as that described above for the analysis of monohydroperoxy-DHA. The same column was also used, and a mobile phase consisting of acetonitrile/aqueous acetic acid (1%) 80:20 (vol/vol) with a flow rate of 1 mL/min allowed DHA to elute after 11 min. DHA was then quantified with a 500 ELSD (Alltech Associates, Deerfield, IL) with a nebulizer gas flow of 2.26 standard liters per minute and a drift tube temperature of 91°C.

LC-MS. The different isomers of monohydroperoxy-DHA were characterized with LC-MS. They were first separated on an HPLC from PerkinElmer (Boston, MA) according to the method described above. One-tenth of the flow from the column was then led into the QSTAR® Q-TOF hybrid tandem mass spectrometer (PE Sciex, Toronto, Canada) with a TurboIonSpray™ source set to negative ion mode. The TOF-MS mode and the product ion mode were set to ranges of *m/z* 70–700 and *m/z* 70–520, respectively.

Oxidation conditions. The stability of DHA with respect to the formation of monohydroperoxy-DHA and polyhydroperoxy-DHA and the reduction of DHA were monitored at 6, 25, and 40°C for a period of 7 d, in two different systems, i.e., bulk and liposomes. DHA bulk (0.12 g) was stored in an open 10-mL vial inside a sealed 500-mL bottle, whereas DHA in liposomes (7 mL) was stored in a sealed 50-mL bottle. Thus, an excess of oxygen was available in the air present in the bottles at the beginning of the stability test, although the bottles were sealed during incubation. Air was also let in during sampling. All samples were shielded from UV light. An additional liposome experiment with 0.1 mM $FeSO_4 \cdot 7H_2O$ incubated at 25°C was also performed.

DHA liposomes containing antioxidants incubated at 40°C were then investigated with respect to resistance to oxidation for a period of 7 d. The antioxidants tested were ascorbic acid (0.5 mM), α-tocopherol (0.5 mM), a combination of ascorbic acid (0.5 mM) and α-tocopherol (0.5 mM), and juice from sea buckthorn. The latter was centrifuged at $11{,}500 \times g$ for 4 min. Part of the oil phase and part of the water phase were withdrawn separately (the precipitate formed was not used). The liposome with sea buckthorn juice contained mainly three important antioxidants, i.e., ascorbic acid (1.6 µg/mL liposome), α-tocopherol (0.13 µg/mL liposome), and quercetin-glucosides (0.15 µg/mL liposome) (6,7). Even though ascorbic acid was the predominant antioxidant in sea buckthorn, the liposome with pure ascorbic acid contained over 50 times more of this antioxidant than the liposome containing sea buckthorn juice.

Each experiment was performed three times, and on each sampling occasion one sample was withdrawn for hydroperoxide measurement and one for DHA content determination. These samples were diluted with acetonitrile when necessary to obtain DHA concentrations of 1.67 mg DHA/mL (for analysis of hydroperoxides) and 0.84 mg DHA/mL (for analysis of DHA content).

Preparation of liposomes. Lecithin, DHA, and a lipophilic antioxidant (α-tocopherol or 23 mg oil phase of the sea buckthorn juice), if used, were dissolved in chloroform, which was then evaporated to obtain a homogeneous starting material for the preparation of liposomes. Water (40 mL) at a temperature of 50°C was added, and the system containing 1.67 mg DHA/mL and 16 mg lecithin/mL was stirred at 700 rpm in a round-bottomed flask in a 50°C water bath for 30 min. The solution was homogenized with a Yellowline DI25 basic disperser (IKA®

Werke GmbH & Co. KG, Staufen, Germany) at 8,000 rpm for 3×5 s. The solution was then stirred at 700 rpm for 65–75 min, while the temperature was maintained at 50°C. To reduce the range of vesicle sizes in the liposome system, the solution was passed 11 times through a 0.1 μm Nuclepore polycarbonate membrane (Whatman, Brentford, United Kingdom) (1 mL at a time) using a mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL) that was heated to approximately 40°C. To prevent oxidation during the preparation of liposomes, nitrogen gas was added several times. When hydrophilic antioxidants [ascorbic acid, the hydrophilic fraction of the sea buckthorn juice (19 μL/7 mL liposome preparation)] were used, they were added after the sample had been withdrawn at time zero.

Evaluation. The unit mol% hydroperoxides was used for both mono- and polyhydroperoxides, and mol% DHA was used for DHA. The former unit corresponds to mol hydroperoxide divided by the amount of pure DHA (mol) expected in an unoxidized sample, expressed as a percentage. The latter unit was defined as the measured DHA content (mol) divided by the measured initial DHA content (mol) obtained as an average of the starting values within an experimental series, expressed as a percentage.

An approximation of the maximum amount of DHA containing hydroperoxides was calculated according to Equation 2. This calculation will give the exact amount of DHA containing hydroperoxides if all polyhydroperoxides contain two hydroperoxy groups. When polyhydroperoxides with more than two hydroperoxide groups are present, the total fraction of DHA containing hydroperoxides will be overestimated.

$$\sum \mathrm{DHA}_{\mathrm{hydroperoxides}} = \sum \mathrm{monohydroperoxides} + \frac{\sum \mathrm{polyhydroperoxides}}{2} \quad [2]$$

Statistics. Three samples were analyzed under each set of experimental conditions. The average of these values without and with a 95% confidence limit was calculated at the starting point and other times in each experiment. The confidence limit is based on the pooled SD for all values after the starting value within an experimental series. When appropriate, whether the difference between two points within one or between two experimental series was significant was tested. The underlying data were tested with a two-sided *t*-test in Microsoft Excel™ (unless stated otherwise) assuming that the values tested had the same variance.

RESULTS

Identification of isomers of monohydroperoxy-DHA. The HPLC method developed provided eight peaks, each of which contained one or two isomers of monohydroperoxy-DHA (Fig. 1). The identification of the isomers is described in the paragraph below. The first peak, with the shortest retention time, corresponded to DHA with a hydroperoxide group on carbon 20 (C20-hydroperoxide). The second peak contained both C17- and C16-hydroperoxides (see Scheme 1; in this scheme each number corresponds to a peak in the chromatogram, and the position of the number indicates the location of the hydroperoxide group in DHA). C13- and C14-hydroperoxides were identified in the third peak. The fourth, fifth, sixth, and seventh peaks contained DHA with a hydroperoxide on carbons 10, 11, 7, and 8, respectively. The last peak corresponded to the C4-hydroperoxide together with a minor contribution from the C5-hydroperoxide. When a hydroperoxide is incorporated at a double bond of the DHA molecule, this bond will move one step in the carbon chain and a conjugated diene is usually formed. Our method, which was highly sensitive to conjugated dienes because a wavelength of 234 nm was used, thus shows a high response to all the hydroperoxides except those not containing a conjugated diene, i.e., hydroperoxides situated at carbons 3, 5, 19, or 21. Only the C5-hydroperoxide was found using LS-MS.

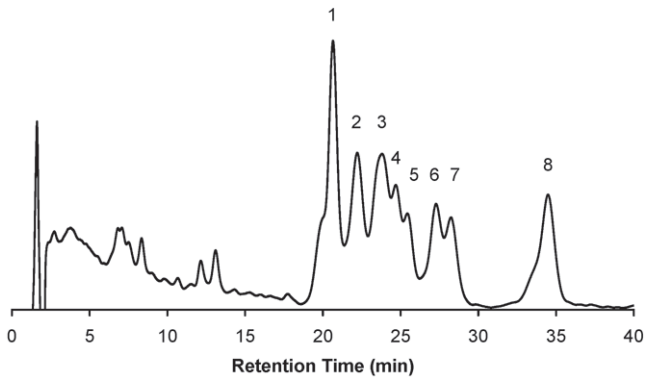
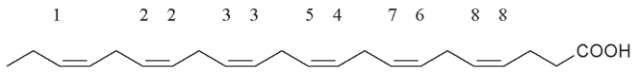
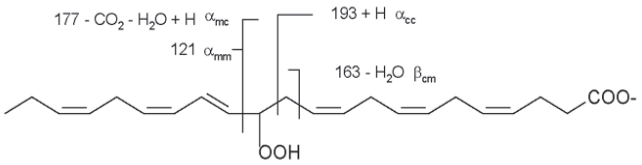


FIG. 1. Different isomers of monohydroperoxy-DHA can be seen in the chromatogram. The range in retention time for the peaks was 19–36 min, where Peak 1 has the shortest retention time. This sample was a bulk DHA incubated at 6°C for 3 d.

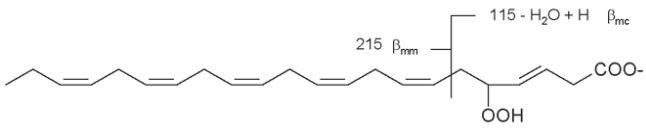
To identify the various monohydroperoxy-DHA isomers, fragments were created using Q-TOF tandem MS. Some fragments could be identified by the characteristic location of the hydroperoxide group; that is, the DHA molecule was cleaved adjacent to the hydroperoxide, and the characteristic fragment could then be identified as the part not containing the hydroperoxide (8). When the cleavage of the carbon chain was toward the carbonyl end (c) or the methyl end (m), the fragment will be defined below as an α_{cc}-fragment or α_{mm}-fragment, respectively (Scheme 2, fragments created from the C13-hydroperoxide). (The first letter in the subscript defines the cleavage position, whereas the second defines whether the fragment contains the carboxyl or methyl side of the monohydroperoxy-DHA isomer.) Other characteristic fragments are created when cleavage takes place one carbon atom away from the hydroperoxide carbon (8). The characteristic fragment containing the functional group will lose a water molecule and be transformed into a hy-



SCHEME 1



SCHEME 2



SCHEME 3

droxide, and a double bond is then formed between the cleavage carbon and the carbon of the functional group. When the cleavage within the carbon chain is toward the carbonyl end or the methyl end, the fragment is denoted below as β_{cm} or β_{mc}, respectively (see Schemes 2 and 3, the latter indicating fragments created from the C5-hydroperoxide). The characteristic fragments of the different isomers of monohydroperoxy-DHA in the eight peaks of the HPLC chromatogram are presented in Table 1. Besides α_{cc}-, α_{mm}-, β_{cm}-, and β_{mc}-fragments, α_{mc}- and β_{mm}-fragments also were found. Some fragments created were common to all of the isomers and thus did not give any further information on where the hydroperoxide was situated. Such fragments were $[M - H - H_2O]^-$ at *m/z* 341 and $[M - H - H_2O - CO_2]^-$ at *m/z* 297, which were created when water or both water and carbon dioxide were lost from the molecule, respectively.

TABLE 1
Identification of Monohydroperoxy-DHA Isomers with Characteristic Fragments Generated by Q-TOF Tandem MS[a]

Peak	Location of hydroperoxide (carbon atom)	Characteristic fragments (*m/z*)
1	20	285 (α_{cc}), 241($\alpha_{cc} - CO_2$)
2	17	245 (α_{cc}), 111 (β_{cm} + H), 201 ($\alpha_{cc} - CO_2$)
2	16	233 (α_{cc}), 123 (β_{cm}), 189 ($\alpha_{cc} - CO_2$)
3	14	205 (α_{cc}), 151 (β_{cm} + H), 189 (α_{mc})
3	13	193 (α_{cc}), 163 (β_{cm}), 121 (α_{mm}), 177 (α_{mc})
4	10	161 (α_{mm}), 203 (β_{cm}), 151 ($\beta_{mc} - CO_2$)
5	11	121 ($\alpha_{cc} - CO_2$), 163 ($\beta_{mc} - CO_2$)
6	7	201 (α_{mm}), 243 (β_{cm})
7	8	189 (α_{mm}), 123 ($\beta_{mc} - CO_2$)
8	5	115 (β_{mc}), 215 (β_{mm})
8	4	241 (α_{mm})

[a]Q-TOF, quadrupole time-of-flight.

Oxidation of bulk DHA at three temperatures. The total amount of monohydroperoxy-DHA in bulk DHA incubated at 6, 25, and 40°C during a period of 7 d is presented in Figure 2. Although the monohydroperoxy-DHA formation was slowest at 6°C, this temperature gave rise to the highest amount of monohydroperoxy-DHA over the 7 d (9 mol%, day 4). The samples maintained at 25 and 40°C exhibited nearly the same initial formation rate of monohydroperoxy-DHA. However, the 40°C sample reached a peak of 4 mol% at 12 h, while the monohydroperoxy-DHA of the 25°C sample continued to increase for another 12 h, reaching 7 mol%. After the highest amount of monohydroperoxy-DHA was reached at the three temperatures, the monohydroperoxides decreased. This indicates that the monohydroperoxy-DHA continued to be oxidized at a higher rate than the rate at which hydroperoxide was incorporated into pure DHA.

The contribution of the eight monohydroperoxy-DHA isomers, detected with our HPLC method, to the total amount of monohydroperoxides varied. During the increase in the total amount of monohydroperoxides at 6 and 25°C (Fig. 2), the C4-monohydroperoxide increased most: 7–8% relative to the other isomers (Fig. 3A). When the temperature was increased to

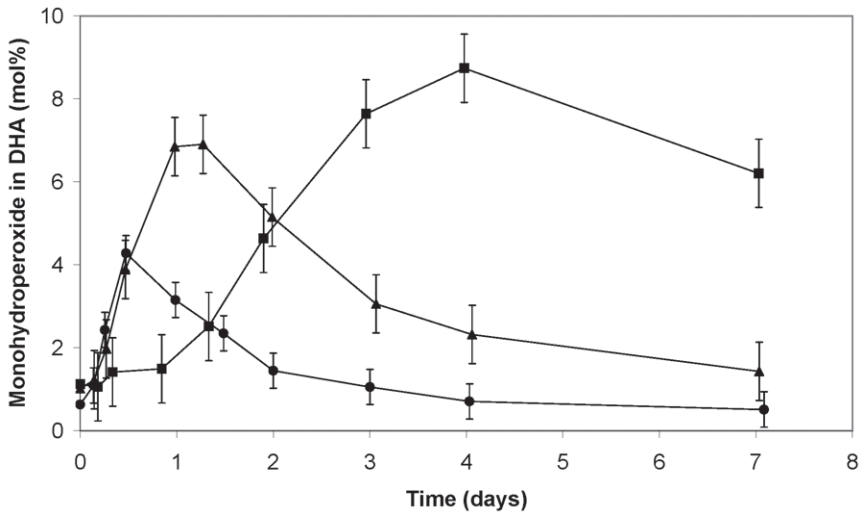


FIG. 2. Total amount of monohydroperoxides in bulk DHA at 6 (■), 25 (▲), and 40°C (●).

40°C, this isomer increased most only during the first 3 h (11% relative to the other 10 isomers, Fig. 3B). During the decrease in the total amount of monohydroperoxides, the C4-monohydroperoxide decreased most relative to the other isomers at all three temperatures, which was probably caused by further oxidation. However, the predominant isomer at all temperatures was the C20-monohydroperoxide. This isomer increased by 3, 17, and 18% at 6, 25, and 40°C, respectively, relative to the other isomers over the first 4 d. The increase continued at the two lower temperatures, while a decrease (6%) was observed at 40°C. Thus, the C20-monohydroperoxide decreased at the slowest rate during the decrease in the total amount of monohydroperoxides, according to Equation 3. (FI_x and CI_x are defined as "formation of isomer *x*" and "conversion of isomer *x* to another compound," respectively.) This isomer had either the highest formation rate (term 1) or the slowest conversion rate to another compound such as a polyhydroperoxide (term 2) during the decrease in the total amount of monohydroperoxides. A combination of both explanations is also possible.

$$\frac{\Delta \text{isomer}_x}{\Delta t} = \frac{\Delta FI_x}{\Delta t} - \frac{\Delta CI_x}{\Delta t} \quad [3]$$

The initial formation rate of polyhydroperoxides in DHA was lowest at 6°C and higher at 25 and 40°C (Fig. 4). The polyhydroperoxides in the 6°C sample increased during the 7 d and reached 10 mol%. The polyhydroperoxides in the 40°C sample increased for the first 24 h. The level reached was thereafter fairly constant. The polyhydroperoxide in the 25°C sample increased further and leveled out after 3 d. The levels of polyhydroperoxide in the 25 and 40°C samples after 7 d were 7 and 5 mol%, respectively, i.e., a significant difference ($P < 0.001$).

The DHA content decreased with time at all temperatures (Fig. 5). The 6°C sample exhibited the slowest reduction rate, which was maintained during the 7 d, with the DHA content finally reaching 15 mol%. The initial reduction rate increased with temperature, and the highest degradation occurred at

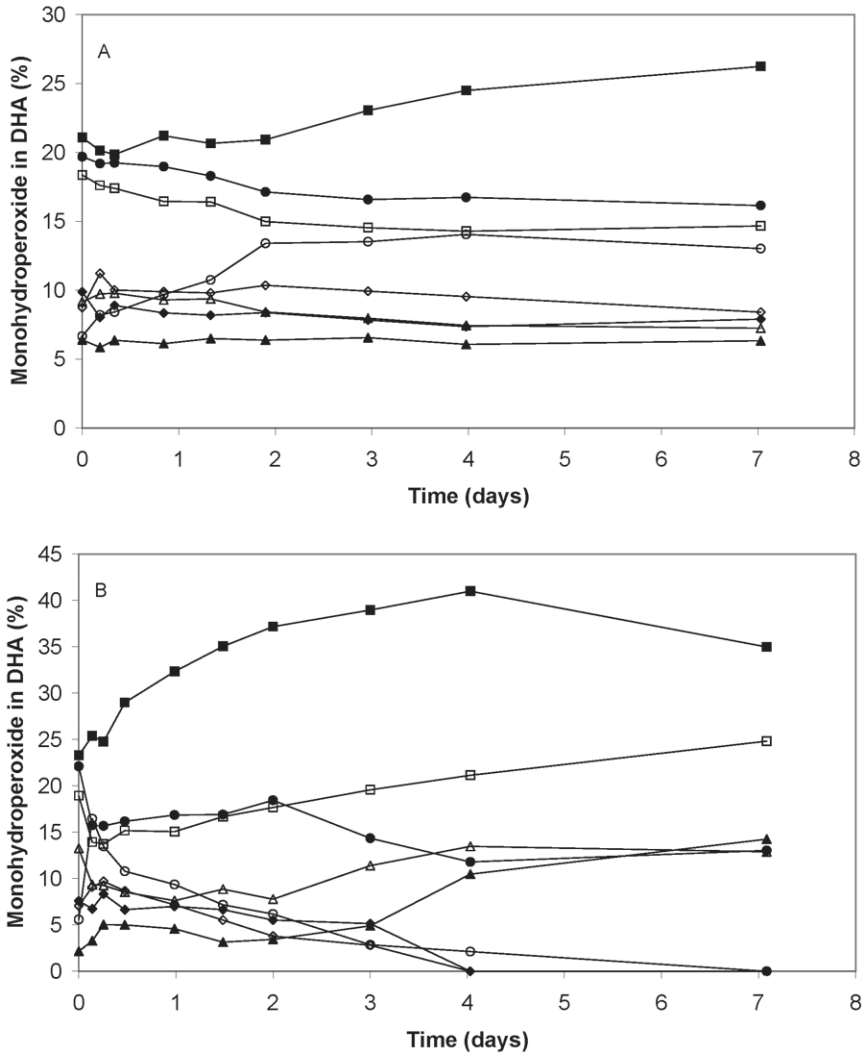


FIG. 3. The isomer profile of bulk DHA at 6 (A) and 40°C (B). C20 (■), C16 + C17 (□), C13 + C14 (●), C10 (△), C11 (▲), C7 (◇), C8 (◆), and C4 (○).

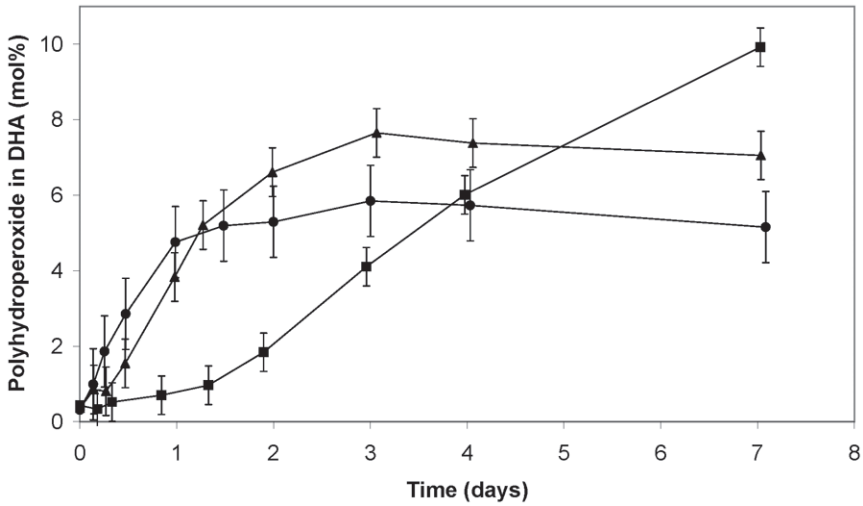


FIG. 4. Total amount of polyhydroperoxides [hydroperoxide groups (mol) in polyhydroperoxides/amount (mol) of DHA in an unoxidized sample] in bulk DHA at 6 (■), 25 (▲), and 40°C (●).

40°C. When approximately 30 mol% DHA was left in the samples incubated at 25 and 40°C, the degradation rate slowed, and after 7 d 14 and 4 mol% DHA was left, respectively. The fraction of DHA that was converted to mono- and polyhydroperoxides (according to Eq. 2) after 7 d was, at most, 11, 5, and 3 mol% at 6, 25, and 40°C, respectively. Thus, a larger proportion of DHA was converted to other oxidation products than hydroperoxides, measured with our HPLC method. Since the DHA became more viscous with time, it is probable that one of these oxidation products was polymerized DHA.

Oxidation of DHA in liposomes at three temperatures. Oxidation of DHA in liposomes was monitored with respect to both hydroperoxide formation and reduction in DHA content. The lecithin used to prepare the liposomes contained linoleic acid and a small amount linolenic acid according to the lecithin supplier. Thus, the hydroperoxides found in this study were generated not only from DHA but also from linoleic acid. Unfortunately, the monohydroperoxides of linoleic acid showed three peaks (two contained double peaks), which interfered with the measurement of the first five monohydroperoxy-DHA peaks eluted in the chromatogram. These peaks were therefore overestimated with respect to the monohydroperoxy-DHA isomers. The polyhydroperoxides arose from oxidation of both DHA and linoleic acid.

The total amount of monohydroperoxides in liposomes containing DHA incubated at 6, 25, and 40°C is presented in Figure 6. The sample at 6°C was quite resistant to oxidation with respect to monohydroperoxides, which increased only by 0.4 mol% (which was, however, a significant increase according to the one-sided *t*-test, $P < 0.0001$). When the temperature was

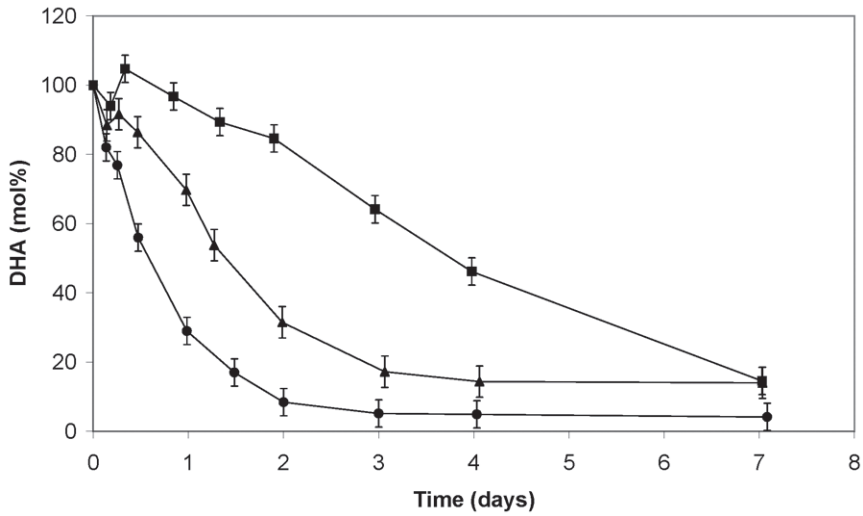


FIG. 5. The relative DHA concentration of bulk DHA at 6 (■), 25 (▲), and 40°C (●).

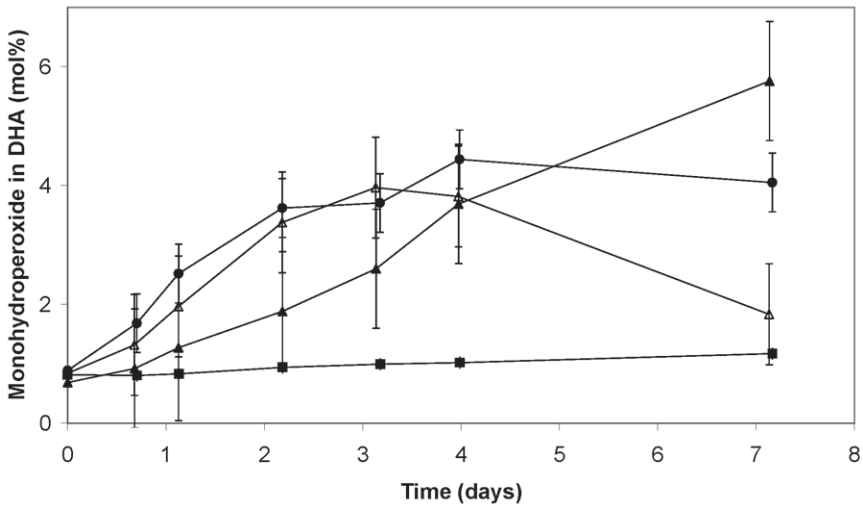


FIG. 6. Total amount of monohydroperoxides in liposomes without $FeSO_4$ at 6 (■), 25 (▲), and 40°C (●) and with $FeSO_4$ at 25°C (△).

increased to 25°C, the monohydroperoxides increased to 6 mol% after 7 d. At 40°C the initial oxidation rate was higher, and a peak (4 mol%) was exhibited after 4 d. When a liposome sample containing both DHA and 0.1 mM $FeSO_4$ was incubated at 25°C, the increase in monohydroperoxides was similar to that in the liposome incubated at 40°C during the first 3 d. Then the monohydroperoxides decreased more rapidly, and the monohydroperoxide level of the liposome containing iron and the liposome incubated at 40°C differed significantly ($P < 0.02$) at day 7.

Among the monohydroperoxy-DHA isomers the monohydroperoxide on C4 and on C20 exhibited the most distinct trends. Initially, the C4-monohydroperoxide increased most relative to the other isomers. At 6°C the C4 isomer increased relative to the other isomers over the 7 d, while a peak was reached after 2 d and 1 d at 25 and 40°C, respectively. The peak (data not shown) containing both the C20-monohydroperoxy-DHA isomer and a hydroperoxy isomer in linoleic acid exhibited the highest relative contribution to the total amount of monohydroperoxides. This peak increased with both time and temperature.

With respect to polyhydroperoxide formation, the liposome incubated at 6°C was fairly stable (data not shown), since the polyhydroperoxides only increased from 1.2 to 1.8 mol% after 7 d (this was, however, a significant difference, $P < 0.01$). The polyhydroperoxides increased with time and temperature. After 7 d at 25 and 40°C, 25 and 35 mol% polyhydroperoxides were observed, respectively. Addition of 0.1 mM $FeSO_4$ to a liposome solution incubated at 25°C led to a polyhydroperoxide formation profile similar to that of the liposomes stored at 40°C without any $FeSO_4$. A two-sided *t*-test at the 95% confidence level rejected a difference between the samples at all points in time.

The DHA content of the liposomes is shown in Figure 7. The liposome incubated at 6°C was stable, since the minor re-

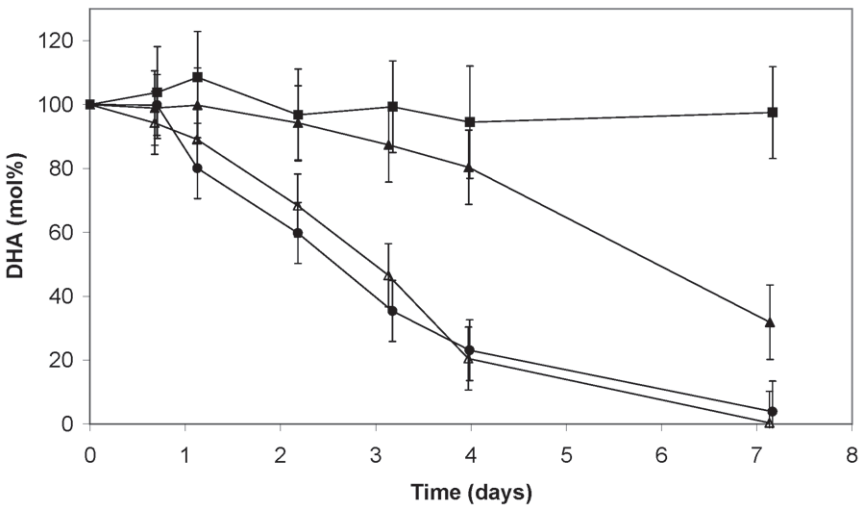


FIG. 7. The relative DHA concentration of DHA in liposomes without $FeSO_4$ at 6 (■), 25 (▲), and 40°C (●) and with $FeSO_4$ at 25°C (△).

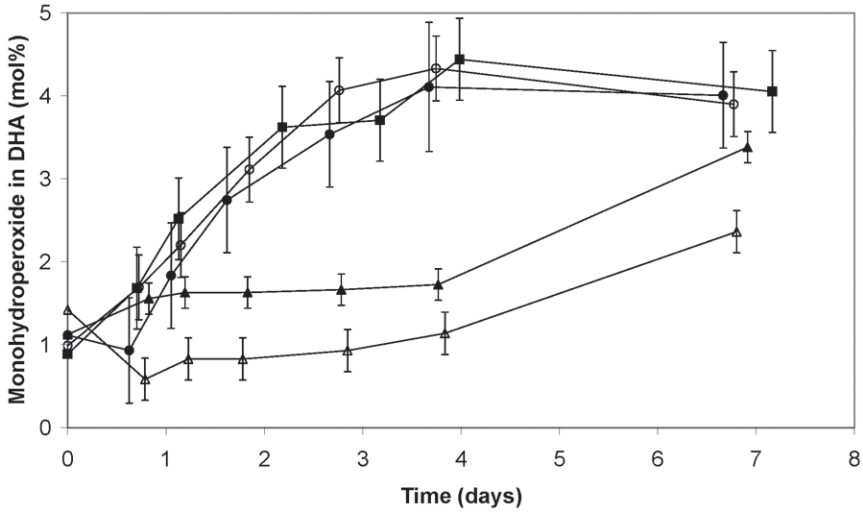


FIG. 8. Total amount of monohydroperoxides in liposomes without antioxidants (■) or with antioxidants: ascorbic acid (●), α-tocopherol (▲), α-tocopherol + ascorbic acid (△), and sea buckthorn juice (○).

duction of DHA was not significant. The DHA content decreased with time and temperature as shown in Figure 7. After 7 d of storage, 31 and 0.4 mol% DHA remained at 25 and 40°C, respectively. Addition of $FeSO_4$ to a liposome stored at 25°C led to a DHA content profile similar to that of the liposome at 40°C without any $FeSO_4$. A two-sided *t*-test at the 95% confidence level of the samples rejected a difference between all sampling times except the last. The maximal proportions of DHA converted to monohydroperoxides and polyhydroperoxides were only 18 and 21 mol% at 25 and 40°C, respectively, over 7 d. Thus, a large part of the DHA was converted to products other than the hydroperoxides as measured with our HPLC method at these two temperatures.

Oxidation of DHA in liposomes after addition of antioxidants. The amount of monohydroperoxides in samples containing antioxidants incubated at 40°C is presented in Figure 8. Addition of α-tocopherol (0.5 mM) reduced the oxidation to monohydroperoxides during the first 4 d. Then the monohydroperoxides increased, reaching 3.4 mol%. A two-sided *t*-test at the 95% confidence level showed that there was a significant difference between the reference and liposome containing α-tocopherol on day 4, while a difference on day 7 was rejected. Addition of both α-tocopherol and ascorbic acid reduced the oxidation to monohydroperoxides even more; the difference according to the *t*-test was significant at the 95% confidence level. It should be noted that the percentage of C4-monohydroperoxide was quite stable in both experiments over the 7 d (data not shown). Addition of only ascorbic acid or juice from sea buckthorn did not lead to any reduction in the monohydroperoxides, which was supported by a two-sided *t*-test at the 95% confidence level.

Addition of α-tocopherol reduced the formation of polyhydroperoxides considerably (data not shown). The combination of α-tocopherol and ascorbic acid showed a similar pattern, and a difference between the liposome containing only α-tocopherol and the combination of α-tocopherol and ascorbic acid was rejected, according to a two-sided *t*-test at a confidence level of 95%, during the first 4 d. However, on day 7, the liposome with the vitamin combination showed a lower amount of polyhydroperoxide (12 mol%) than the liposome with α-tocopherol alone (17 mol%) (this differences was significant). Addition of ascorbic acid or sea buckthorn juice provided no protection to DHA in liposomes with respect to the formation of polyhydroperoxides, and levels of 32 and 34 mol% were reached, respectively, after 7 d of incubation at 40°C.

The relative DHA content of the liposomes is shown in Figure 9. Addition of α-tocopherol almost completely prevented the degradation of DHA during the first 4 d. Then, on day 7 the DHA content was reduced to 58 mol%. When the combination of α-tocopherol and ascorbic acid was added, the DHA content was reduced slowly and reached 70 mol% after 7 d. A significant difference between the two samples based on a *t*-test at the 95% confidence level was observed after 2 d. The ascorbic acid seemed to protect the DHA during the two first days. The DHA content then decreased considerably and was as degraded as the reference. The DHA degradation was not reduced when juice from sea buckthorn was added. Since the level of hydroperoxide formed in liposomes when α-tocopherol was added corresponded to the reduction in DHA during the first 4 d, significant amounts of other oxidation products were not formed. DHA was then degraded further to other oxidation products as well. The liposomes containing other additives were degraded to other oxidation products earlier.

DISCUSSION

The analysis method developed allowed quantification of eight isomer groups of monohydroperoxy-DHA, and showed that these oxidation products initially increased faster in the bulk system than in the liposome system. The C20-monohydroper-

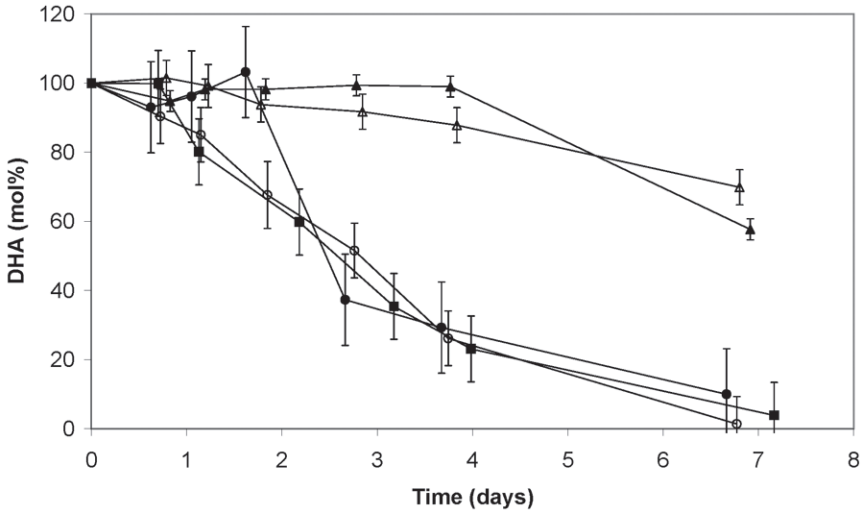


FIG. 9. The relative DHA concentration of liposomes without antioxidants (■) or with antioxidants: ascorbic acid (●), α-tocopherol (▲), α-tocopherol + ascorbic acid (△), and sea buckthorn juice (○).

oxy-DHA provided the largest contribution to the total amount of monohydroperoxides and showed a rapid increase, thus demonstrating that the double bond farthest from the carboxyl group was highly sensitive to oxidation. Although all isomers decreased after a peak in the total amount of monohydroperoxides was reached, this isomer continued to increase relative to the other isomers. This could be interpreted either as further degradation of this isomer at a slower rate than the other isomers, or a continued increase in this isomer at a higher rate than the other isomers, although the degradation rate was higher than the formation rate. Furthermore, the C4-monohydroperoxide, which is situated on the double bond nearest the carboxyl group, was sensitive to oxidation and showed behavior that deviated from that of the other isomers. The C4-monohydroperoxide increased considerably during the initial increase in the total amount of monohydroperoxides. However, when the maximum of the total amount of monohydroperoxides was reached, this isomer decreased faster than the other isomers, indicating that it was most sensitive to further oxidation. The other isomers, i.e., a hydroperoxide group on either carbon 17, 16, 14, 13, 11, 10, 8, or 7, increased to a lesser extent, indicating that the second to fifth double bonds within the DHA molecule were less sensitive to oxidation. However, the hydroperoxides at double bonds two to five are highly prone to further oxidation to hydroperoxy epidioxides and hydroperoxy bicycloendoperoxides (1). These oxidation products cannot be formed from the hydroperoxide groups situated on carbons 20 and 4, since they are located outside the group of double bounds. This may explain why the formation of C20 and C4 isomers was most extensive. Double bonds two to five were probably oxidized more than the detected monohydroperoxides, and the hydroperoxy epidioxides and hydroperoxy bicycloendoperoxides formed were instead detected as polyhydroperoxides. Our findings are supported by another study in which the DHA ethyl ester incubated in chloroform with a radical inducer at 37°C exhibited the highest hydroperoxide formation at carbon 20 and carbon 4 (9). However, when the DHA ethyl ester was incubated in an aqueous emulsion instead, the hydroperoxide formation at carbons 16, 4, 20, and 10 was highest. This result was explained by the epidioxide formation being slower at carbons 16 and 10 and/or that the oxygen attack was greater at these positions in the emulsion system.

Our method also revealed the profile of the total amount of monohydroperoxides with time, which increased most initially, and then decreased in the bulk system to the benefit of other oxidation products. At the lowest temperature investigated, i.e., 6°C, the amounts of monohydroperoxides and polyhydroperoxides found were quite low and stable in the liposome system, whereas these oxidation products increased much more in the bulk system at this temperature. In the liposome system the polyhydroperoxides increased considerably with temperature and time. In contrast, the polyhydroperoxides initially increased fast at 25 and 40°C in the bulk system. A peak was reached and then the polyhydroperoxides slowly decreased. This is probably caused by a considerable amount of polymerization, which was visually observed in the bulk system.

The measured DHA content confirmed that the liposome system containing DHA incubated at 6°C was stable, whereas only 15 mol% DHA was left in the bulk system after 7 d at the same temperature. The degradation increased with temperature and time for both the liposome and bulk systems. Thus, the liposome system provided better protection of DHA than the bulk system, although at a temperature of 25°C and above there was considerable degradation of DHA. Other studies also have shown that liposomes provide better protection against oxidation than a bulk system when the PUFA are incorporated into either PC or TG (10,11). When DHA was dissolved in acetonitrile at the same concentration as in the liposome system, the DHA was found to degrade at a slower rate (data not shown). Only 10% degradation of DHA was found after 4 d of incubation at 6, 25, or 40°C.

The addition of α-tocopherol to the DHA liposomes incubated at 40°C resulted in a considerable reduction in mono- and polyhydroperoxide formation together with slower degradation of unoxidized DHA. This vitamin is lipophilic and therefore probably evenly distributed among the DHA molecules in the liposome structure. The protection offered was greatest during the first 4 d. On day 7 the rate of oxidation was seen to increase, indicating that most of the α-tocopherol had been consumed. Addition of both α-tocopherol and ascorbic acid to the DHA liposome produced lower degradation on day 7, which has been explained by the α-tocopherol radical being regenerated by the ascorbic acid (1). Addition of only ascorbic acid provided no protection of the DHA liposome. Thus, ascorbic acid acted as neither an antioxidant nor a pro-oxidant. However, the latter effect may be induced by the addition of iron, since ascorbic acid can reduce Fe^{3+} to Fe^{2+}, which can decompose hydroperoxides (12). *In vivo*, iron is bound to metal proteins, and therefore the pro-oxidant effect of ascorbic acid may be eliminated (12). The fact that the sea buckthorn juice provided no protection of DHA in the liposome system may be explained by the low level of antioxidants. Even though sea buckthorn juice is rich in ascorbic acid, the liposome with sea buckthorn juice contained 50 times less ascorbic acid than the liposomes with pure ascorbic acid. The other antioxidants, α-tocopherol and quercetin-glucosides, provided an even lower concentration in the liposomes containing sea buckthorn juice.

Compared with currently available methods for analyzing primary oxidation products of PUFA, i.e., hydroperoxides, this method offers the advantage of differentiating between several monohydroperoxy-DHA isomers without requiring any derivatization step prior to analysis. For example, when GC was used to separate the isomers (3), the FA had to be derivatized prior to the analysis. Thus, this method may give misleading results due to either the derivatization step or the high temperature used during the measurements, both of which can affect the fragile hydroperoxide. In another study, thermospray LC-MS was used with a gradient of methanol and aqueous ammonium acetate to separate the isomers (4). Although the mobile phase and the column were not the same as those used in our method, the isomers were found to elute in the same order, but a longer analysis time was needed. A simplified HPLC method for analyzing the total amount of hydroperoxides and the reduction of unoxidized PUFA such as DHA also has been reported (13). The total amount of hydroperoxides can also be estimated by determining the peroxide value by titration or spectrophotometric measurements (1).

Our method thus provides a straightforward and accurate means of determining which part of DHA is most vulnerable to hydroperoxide formation. Double bonds 6 and 1 from the carboxyl group must be protected to reduce hydroperoxide formation. Since the other bonds probably form hydroperoxy epidioxides and hydroperoxy bicycloendoperoxides, these bonds should also be protected. One proposed strategy is to add antioxidants, such as the combination of α-tocopherol and ascorbic acid (investigated above), or squalene, which was discovered to have a protective effect on DHA (13). Another option might be to incorporate the DHA molecule into a lipid molecule such as a phospholipid (14). Hydroperoxide formation was completely prevented at carbons 4, 7, 8, and 11 when DHA was incorporated at one position of either PC or PE and incubated at 25°C. Furthermore, DHA should be stored in a suitable system, and liposomes were shown to be better than bulk in the current study.

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Lipase-Catalyzed Esterification of 2-Monoricinolein for 1,2(2,3)-Diricinolein Synthesis

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ABSTRACT: The purpose of this investigation was to develop conditions for producing 2-monoricinoleoyl DAG. We used lipase-catalyzed hydrolysis of triricinolein to obtain 2-monoricinolein and thereafter synthesized 1,2(2,3)-diricinolein through esterification of 2-monoricinolein, using ricinoleic acid as the acyl donor. Five different 1,3-specific immobilized lipases were tested for the initial methanolysis reaction: *Candida antarctica* type B, *Rhizomucor miehei*, *Rhizopus oryzae* (ROL), *Thermomyces lanuginosus*, and *Aspergillus niger*. For the second esterification reaction, we investigated these five lipases plus *Pseudomonas cepacia*, *Penicillium roquefortii*, *Candida rugosa*, and *Pseudomonas fluorescence*. Toluene and diisopropyl ether (DIPE) were examined as reaction media at a water activity of 0.11. ROL in DIPE gave the highest yield of 2-monoricinolein from triricinolein, 78% after 3 h of reaction. The isolated 2-monoricinolein was esterified with ricinoleic acid for synthesis of 1,2(2,3)-diricinolein. ROL in DIPE gave the highest yield of 1,2(2,3)-diricinolein, 58% after 1 h of reaction, and NMR analysis showed that the purity was 97.2%. This methodology can be used for synthesizing radiolabeled 1,2(2,3)-diricinolein to study lipid biosynthesis in castor and other oilseeds.

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Castor (*Ricinus communis* L.) seeds contain a highly viscous oil rich in triricinolein, around 71 wt% of the total oil content (1). The ricinoleate content of castor oil is approximately 90 wt%. Ricinoleic acid is a hydroxy FA, which gives the oil its highly viscous properties. Castor oil is an industrially important oil, useful in lubricants and in the production of paints, cosmetics, coatings, and polymers (2). Unfortunately, castor seeds contain ricin, a toxic protein that inhibits protein synthesis in mammalian cells by attacking the ribosome (3). Therefore, work has been done in our laboratory exploring the possibility of producing triricinolein in a plant lacking toxins.

DAG acyltransferase (DGAT) is one of the key enzymes responsible for synthesizing triricinolein from 1,2-diricinolein in castor seeds. In our laboratory, the cDNA for DGAT in castor (RcDGAT) seeds was cloned and expressed in the yeast *Saccharomyces cerevisiae* (4) to produce an enzymatically active protein. To characterize the specific activity of the expressed DGAT enzyme, 1,2-diricinolein was needed as a substrate. In previous work, we described how to produce 1,2(2,3)-diricinolein from triricinolein by methanolysis using *Penicillium roquefortii* lipase (5). However, to further characterize the RcDGAT, it would be helpful to have a source of radiolabeled 1,2-diricinolein to broaden our understanding of the RcDGAT substrate specificity.

Only glycerol and ricinoleic acid are available for purchase as radiolabeled compounds; hence, only a few options exist for lipase-catalyzed synthesis of radiolabeled 1,2(2,3)-diricinolein. The approach is complicated by the need to esterify a hydroxy FA in a reaction for DAG synthesis. A key advantage of using an enzymatic approach instead of chemical radiolabeling is the elimination of side reactions, and there is no need for protecting groups with the choice of a suitable lipase and appropriate reaction conditions. The first option is to esterify radiolabeled glycerol with ricinoleic acid to form 1,2(2,3)-diricinolein directly. However, this is rather difficult to achieve, since no known lipase specifically catalyzes such a reaction. Generally, the products will be a mixture of 2-MAG, 1,3-DAG, and TAG (6), or if the water content is continuously removed, a high yield of TAG (7). The second option is esterification of radiolabeled glycerol with ricinoleic acid to make triricinolein, followed by hydrolysis/alcoholysis of triricinolein into 1,2(2,3)-diricinolein. This reaction was tried in our laboratory, but the first step of the reaction gave only low yields of triricinolein, even though water was removed using molecular sieves. A possible explanation is the inevitable polymerization reactions of hydroxy FA into estolides (8). A third option is hydrolysis/alcoholysis of triricinolein to 2-monoricinolein, followed by esterification of 2-monoricinolein with radiolabeled ricinoleic acid. The first step of the reaction can be optimized to give high yields using 1,3-specific lipases, and the second step can be controlled using a one-to-one molar fraction of ricinoleic acid to 2-monoricinolein. This is the approach that was determined to be the most suitable for our purposes. Hence, in this study we have developed a method for synthesizing 1,2(2,3)-diricinolein involving methanolysis of triricinolein to 2-monoricinolein followed by esterification of 2-monoricinolein with ricinoleic acid.

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Abbreviations: ANL, *Aspergillus niger* lipase; CALB, *Candida antarctica* lipase type B; CRL, *Candida rugosa* lipase; DGAT, diacylglycerol acyltransferase; DIPE, diisopropyl ether; MTBE, methyl *tert*-butyl ether; PCL, *Pseudomonas cepacia* lipase; PFL, *Pseudomonas fluorescence* lipase; PRL, *Penicillium roquefortii* lipase; RcDGAT, the cDNA for DGAT in castor; RML, *Rhizomucor miehei* lipase; ROL, *Rhizopus oryzae* lipase, TLL, *Thermomyces lanuginosus* lipase.

EXPERIMENTAL PROCEDURES

HPLC system. A Waters Millenium HPLC system consisting of a computer, a model 600 gradient pump, a model 717 autosampler, and a model 2487 UV-vis detector (Waters Associates, Milford, MA) was used. A Phenomenex Luna column, CN 5 μ, 250 × 4.6 mm (Phenomenex, Torrance, CA), was used for normal-phase HPLC analysis, a Phenomenex Luna column, C18(2) 100 Å, 5 μ, 250 × 15 mm, was used for preparative chromatography of castor oil, and a Phenomenex Luna column, CN 10 μ, 250 × 10 mm, was used for normal-phase preparative chromatography of monoricinolein.

Solvents. HPLC-grade 2-propanol, toluene, methanol, and hexane were obtained from Fisher Scientific (Fair Lawn, NJ), diisopropyl ether (DIPE) was purchased from Fluka Chemie (Buchs, Germany), and methyl *tert*-butyl ether (MTBE) was obtained from Aldrich (Milwaukee, WI).

Chemicals. Accurel MP1000 was obtained from Membrana (Obernburg, Germany). Molecular sieves (3 Å, 8–12 mesh), lithium chloride, and methyl ricinoleate were purchased from Sigma (St Louis, MO). Sodium sulfate and magnesium nitrate were obtained from J.T.Baker Inc. (Philipsburg, NJ). Potassium sulfate, sodium phosphate, and disodium phosphate were purchased from Spectrum Chemical MFG Corp. (Gardena, CA).

Enzymes. Crude lipases from *Pseudomonas cepacia* (PCL), *Rhizopus oryzae* (ROL), *Thermomyces lanuginosus* (TLL), *Aspergillus niger* (ANL), *Candida rugosa* (CRL), *Pseudomonas fluorescens* (PFL), and *P. roquefortii* (PRL) were kind gifts from Amano Enzymes Inc. (Nagoya, Japan). Novozyme 435 [immobilized *Candida antarctica* lipase type B (CALB), 10.000 propyl laurate units/g] and Lipozyme RM IM [immobilized *Rhizomucor miehei* lipase (RML), 6.1 batch acidolysis units Novo (BAUN)/g] were generous gifts from Novozymes North America (Franklinton, NC). The activity of Novozyme 435 is based on ester synthesis from 1-propanol and lauric acid at 60°C for 15 min. The ester formation is calculated based on the acid values of the reaction mixture measured by titration before and after reaction, and given as propyl laurate units/g. BAUN is based on acidolysis of high oleic acid sunflower oil and decanoic acid at 70°C for 60 min. The rate of the reaction is determined by measuring the amount of decanoic acid incorporated into the 1- and 3-positions of the TAG.

Preparation of triricinolein. Triricinolein is not commercially available, but was produced in our laboratory by preparative chromatography from castor oil, which is composed of 71% triricinolein plus diricinoleins (1). To obtain triricinolein, 0.5 mL of castor oil (0.5 g/mL in 2-propanol) was injected onto the preparative column. The mobile phase used was a gradient of methanol (A) and 2-propanol (B), and the flow rate was 7 mL/min, eluted with 100% A for 5 min, a linear gradient of A/B (80:20) over 10 min, and held for 5 min. The column was then reconditioned back to 100% A over 5 min and held for 5 min. Triricinolein was collected at a retention time of 10 min. The solvent was removed under nitrogen and mild heating, and the purity of the fractions was checked by analytical HPLC.

Immobilization of lipases. Crude lipases were immobilized on a polypropylene powder (Accurel MP1000, formerly called EP100) using adsorption, as described by Gitlesen *et al.* (9). Crude enzyme (5 g) was mixed with 100 mL of phosphate buffer (pH 6.0, 20 mM) and then added to 5 g of Accurel MP1000 that had been prewetted with 15 mL of ethanol. The mixture was incubated overnight at room temperature while stirring. The following day the mixture was filtered, and the filtrate was washed with 5 mL of phosphate buffer (pH 7.0, 200 mM). The immobilized enzyme was dried overnight under vacuum. The activity of the lipases was determined by mixing 40 mg of triolein with 2.5 mL of DIPE (a_w = 0.11) and 20 μL of methanol (a_w = 0.11) and 50 mg of immobilized lipase (a_w = 0.11). Several fractions were taken at 10–30 min intervals, up to 3 h total reaction time. The enzyme activity was expressed as milligrams triolein consumed per gram of enzyme per hour of reaction time. The hydrolytic activities for the enzymes tested in this work were: 3.1 (CRL), 10.1 (PRL), 18.5 (ANL), 22.6 (CALB), 34.8 (PCL), 54.4 (PFL), 250.9 (RML), 280.8 (TLL), and 320.3 (ROL).

Equilibration of the reaction system. Immobilized enzymes, reaction media (toluene, hexane, and DIPE), and methanol were allowed to equilibrate for at least 48 h in airtight desiccators to a water activity (a_w) of 0.11 over saturated LiCl solution (10).

HPLC analysis. The expected products of the lipase reactions, ricinoleic acid, methyl ricinoleate, 1,2(2,3)-diricinolein, 2-monoricinolein, and triricinolein, were analyzed by injection of 20 μL on a normal-phase HPLC column using a mobile phase of MTBE (A) and hexane (B). The gradient was as follows: held for 5 min at A/B (30:70), then a gradient to A/B (60:40) in 15 min, held for 5 min, then a gradient back to A/B (30:70) in 1 min, and held for 9 min. The total run time was 35 min. The flow rate was 1.0 mL/min, with detection at 205 nm.

Methanolysis reaction. Triricinolein (100 mg) was dissolved in 2.5 mL of reaction media and 50 μL of methanol. A 100-μL quantity was removed as a first fraction to determine the starting concentration of triricinolein (which was set to 100% yield on the axis of Figs. 3A, 3B). Thereafter, 50 mg of immobilized enzyme was added, and the reaction took place in glass tubes rotated at room temperature. Fractions (100 μL) were removed at time intervals, the solvent of each collected fraction was removed under nitrogen, and 0.5 mL of mobile phase solution [hexane/MTBE (7:3, vol/vol)] was added. The fractions were stored in a freezer at −20°C until HPLC analysis. The reactions were performed in duplicate.

Optimized methanolysis reaction. ROL, DIPE, and methanol were pre-equilibrated to a_w 0.11 for at least 48 h. Triricinolein (1 g) was dissolved in 10 mL of DIPE and 0.5 mL of methanol. ROL (0.5 g) was added, and the mixture was allowed to react for 30 min with stirring at room temperature. The enzyme was removed by filtration at the end of the reaction time. The solvent was removed by nitrogen, and 0.5 mL of hexane/MTBE (7:3) was added.

Fraction collection of monoricinolein. Portions (0.5-mL) of methanolyzed triricinolein were injected onto the normal-phase HPLC preparative column. A mobile phase consisting of hexane

(A) and MTBE (B) was used at a flow rate of 5.0 mL/min. The gradient elution was as follows: held for 5 min at A/B (30:70), then a gradient to A/B (60:40) in 15 min, then held for 5 min, and a gradient back to A/B (30:70) in 1 min, then held for 4 min. The total run time was 30 min. Fractions of 2.5 mL were collected, and those representing the monoricinolein peak eluting at 17 min were pooled. The solvent was removed by nitrogen under mild heating. The monoricinolein oil recovered was stored at –20°C.

Esterification reaction. 2-Monoricinolein (25 mg) and ricinoleic acid (20 mg) (1:1 molar proportion) were dissolved in 2 mL of reaction media (a_w = 0.11). A sample of 100 µL was taken out as a first fraction to determine the starting concentration of 2-monoricinolein (which was set to 100% yield on the axis of Fig. 5). Thereafter, 10 molecular sieve beads for drying and 50 mg of immobilized enzyme were added, and the reaction took place in glass tubes rotated at room temperature. Fractions of 100 µL were taken out at time intervals, the solvent of each collected fraction was removed by nitrogen, and 0.5 mL of mobile phase solution [hexane/MTBE (7:3, vol/vol)] was added. The fractions were stored in a freezer at –20°C until HPLC analysis. The reactions were performed in duplicate.

Optimized esterification reactions. ROL and DIPE were pre-equilibrated to a_w 0.11 for at least 48 h, 25 mg of 2-monoricinolein and 20 mg of ricinoleic acid (1:1 molar proportion) were dissolved in 2 mL of DIPE, 0.5 g of ROL and 10 molecular sieve beads were added, and the mixture was allowed to react for 1 h with stirring at room temperature. The enzyme was removed by filtration at the end of the reaction time. The solvent was removed by nitrogen, and 0.5 mL of hexane/MTBE (7:3, vol/vol) was added.

Fraction collection of 1,2(2,3)-diricinolein. The procedure was the same as for fraction collection of monoricinolein (see above). The 1,2(2,3)-diricinolein peak was collected at a retention time of 12 min. The solvent was removed by nitrogen under mild heating. The 1,2(2,3)-diricinolein oil recovered was stored at –20°C.

NMR spectroscopy. NMR spectra were obtained at 27°C from samples in $CDCl_3$ with TMS as an internal standard on a Bruker model ARX400 spectrometer (Bruker BioSpin Corp., Fremont, CA) at a frequency of 100.62 MHz for carbon and 400.13 MHz for proton. A 30° pulse at a 2.3-s repetition rate was used for carbon, and a 90° pulse at a 7- to 8-s repetition rate was used for protons. The number of attached protons for ^{13}C signals was determined from distortionless enhancement by polarization transfer (DEPT) 90 and DEPT135 assays.

RESULTS AND DISCUSSION

The purpose of this work was to synthesize 1,2(2,3)-diricinolein by incorporating ricinoleic acid into 2-monoricinolein. Since 2-monoricinolein is not commercially available, this compound was enzymatically synthesized from triricinolein. The reactions examined in this work are shown in Figure 1.

Figure 1A shows the lipase-catalyzed methanolysis reaction of triricinolein to produce 2-monoricinolein. To achieve a high

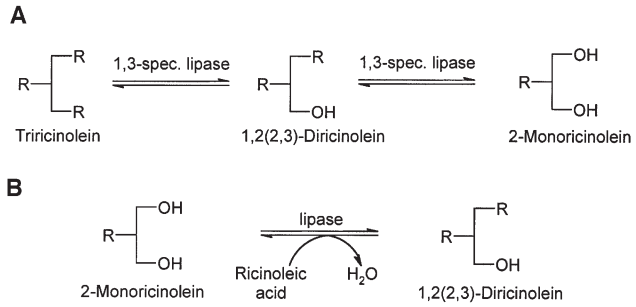

FIG. 1. (A) Reaction scheme for lipase-catalyzed hydrolysis/methanolysis of triricinolein to 2-monoricinolein. (B) Reaction scheme for lipase-catalyzed esterification of 2-monoricinolein and ricinoleic acid to 1,2(2,3)-diricinolein.

yield of 2-monoricinolein (and not complete methanolysis/hydrolysis of triricinolein to glycerol), it is important to use a 1,3-specific lipase. Some of the most highly 1,3-specific lipases are CALB, RML, ROL, TLL, and ANL (11). These were all examined in this study. Figure 1B illustrates the esterification reaction between 2-monoricinolein and ricinoleic acid to produce 1,2(2,3)-diricinolein. In this reaction, one molecule of water is formed for each molecule of diricinolein made by the enzyme. It is important to realize that the water content must be kept at a minimal level; otherwise, the reaction will be driven in the other direction toward hydrolysis. Therefore, molecular sieves were used in all the esterification reactions. Nine different lipases were tested for this reaction, CALB, RML, ROL, TLL, ANL, PCL, PRL, CRL, and PFL.

HPLC analysis. Figures 2A and 2B show examples of chromatography of fractions taken during the methanolysis and esterification reactions, respectively. Complete separation of methyl ricinoleate, ricinoleic acid, triricinolein, 1,2(2,3)-diricinolein, and 2-monoricinolein was accomplished within 30 min of chromatography. Furthermore, 1,2(2,3)-diricinolein was baseline separated from its positional isomer 1,3-diricinolein, although this compound was not formed in any significant amount in any of the reactions.

Optimization of the methanolysis reaction. The conversion of triricinolein to 2-monoricinolein was examined using five different 1,3-specific lipases, CALB, RML, ROL, TLL and ANL. The reactions were carried out in two different solvents, toluene and DIPE. A summary of the results for the methanolysis reaction is shown in Table 1. ROL is clearly the preferred enzyme for catalyzing the desired reaction, giving yields of 2-monoricinolein of approximately 75%. Previous research has shown ROL to be a highly 1,3-specific lipase (12,13).

Figures 3A and 3B show the time course for the ROL-catalyzed reaction in DIPE and toluene, respectively. The reaction was fast in both solvents, as shown by the rapid consumption of triricinolein. However, in DIPE the 2-monoricinolein formed was further converted to glycerol and methyl ricinoleate after its peak at 78% yield. In toluene, the 2-monoricinolein formed was relatively stable for at least 24 h.

The ROL-catalyzed reactions in both DIPE and toluene were scaled up using one gram of triricinolein as the starting

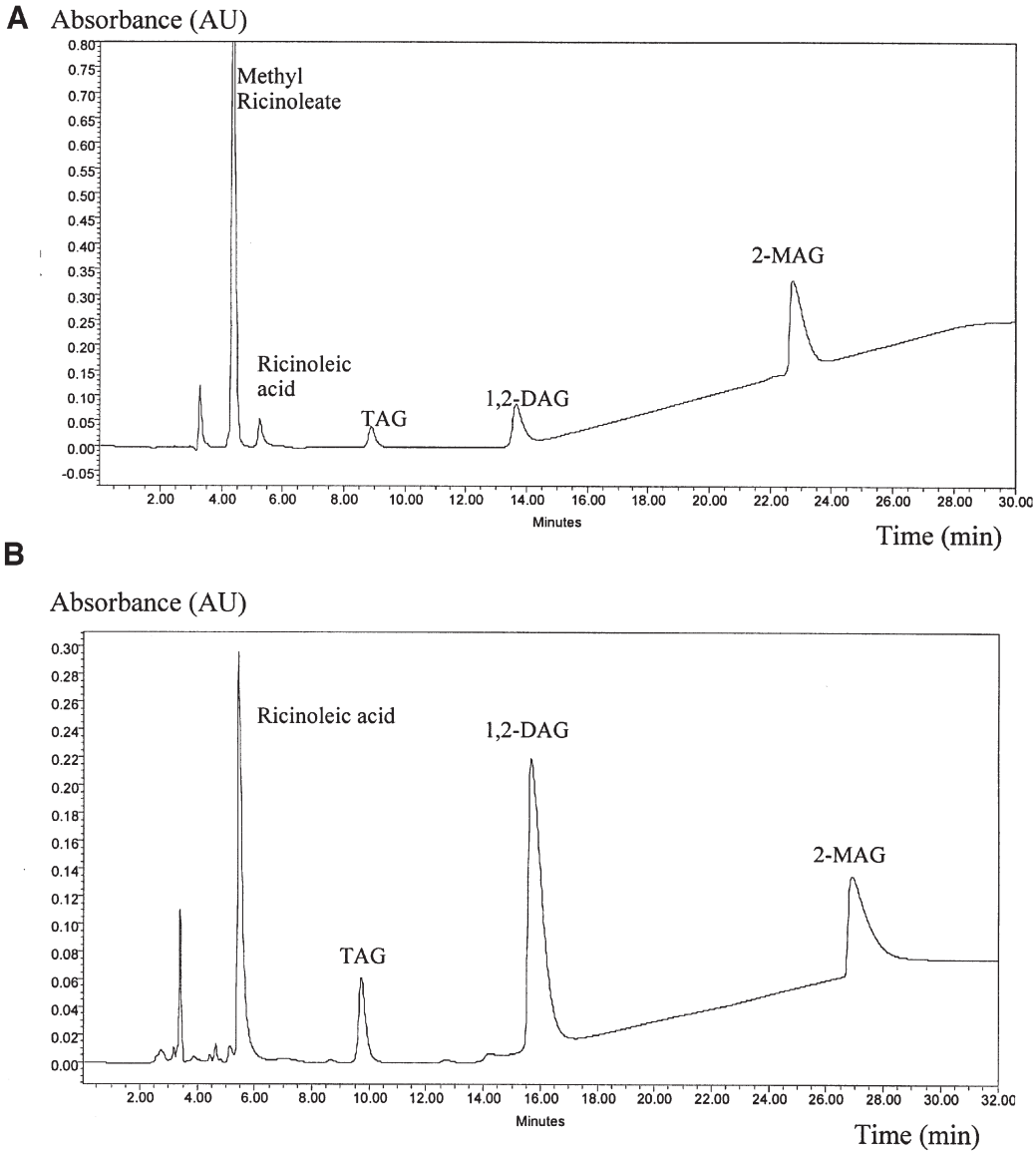

FIG. 2. (A) HPLC of a fraction taken after 1 h of methanolysis reaction of triricinolein using *Rhizomucor miehei* lipase (RML) in diisopropyl ether (DIPE). (B) HPLC of a fraction taken after 30 min of esterification reaction between 2-monoricinolein and ricinoleic acid using *Rhizopus oryzae* lipase (ROL) in DIPE. TAG, triricinolein; DAG, 1,2(2,3)-diricinolein; MAG, 2-monoricinolein.

material. Fractions were taken after certain time intervals. The reaction in toluene was rather slow compared with that in DIPE, requiring more than 24 h of reaction to give approximately 75% yield. The optimized methanolysis reaction was therefore performed in DIPE, giving 78% yield of 2-monoricinolein after only 30 min of reaction. The 2-monoricinolein fraction was collected using a preparative HPLC system (see the Experimental Procedures section). Figure 4 shows a chromatogram from a preparative run of the reaction products obtained using the optimized methanolysis conditions. The 2-monoricinolein was collected from fractions eluting between 16 and 20 min.

The 2-monoricinolein fraction was examined for purity

TABLE 1
Maximum Yields of 2-Monoricinolein (%) from Lipase-Catalyzed Methanolysis of Triricinolein[a]

Lipase	Solvent	
	DIPE	Toluene
CALB	6 (1 h)	1 (48 h)
RML	50 (3 h)	35 (3 h)
ROL	78 (3 h)	75 (3 h)
TLL	47 (1 h)	37 (1 h)
ANL	68 (24 h)	24 (1 h)

[a]Data represent the means of two experiments. The reaction times giving these maximum recoveries are shown within parentheses. ANL, *Aspergillus niger* lipase; DIPE, diisopropyl ether; CALB, *Candida antarctica* lipase type B; RML, *Rhizomucor miehei* lipase; ROL, *Rhizopus oryzae* lipase; TLL, *Thermomyces lanuginosus* lipase.

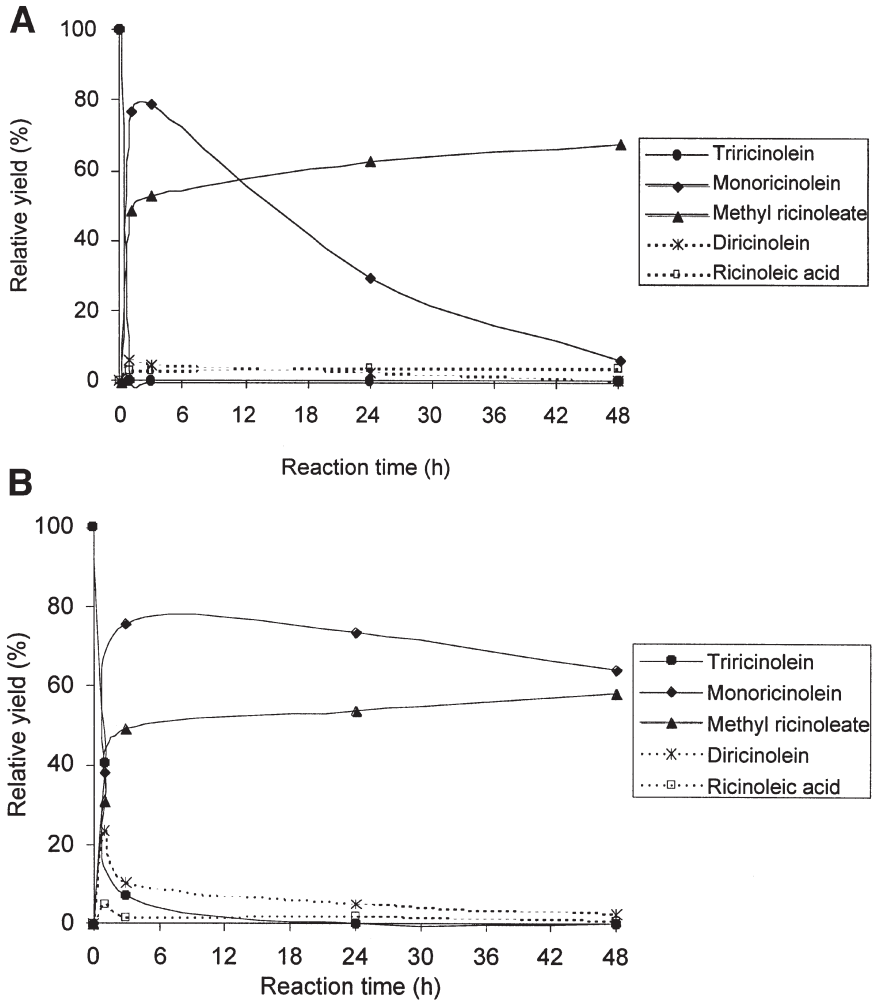

FIG. 3. Time course of the ROL-catalyzed methanolysis reaction of triricinolein in (A) DIPE and (B) toluene. Data points represent the means of two experiments. For abbreviations see Figure 2.

using ^{1}H and ^{13}C NMR. The results showed that the 2-monoricinolein produced approached 100% purity.

Optimization of the esterification reaction. The 2-monoricinolein was esterified with ricinoleic acid to form 1,2(2,3)-diricinolein. Nine different immobilized lipases were examined, CALB, RML, ROL, TLL, ANL, PRL, CRL, PCL and PFL. The reactions were again performed in toluene and DIPE. Table 2 shows the results from all the experiments for this reaction.

As shown in Table 2, RML and ROL in either solvent and TLL in toluene gave high recoveries of 1,2(2,3)-diricinolein, 54–59%. The reaction times giving maximum yields were relatively fast, 0.5–1.5 h. After studying the chromatograms, TLL was excluded as it showed several unknown peaks, which eluted near diricinolein and would thereby contaminate the final fraction of 1,2(2,3)-diricinolein or affect recovery of a pure fraction (data not shown). RML and ROL, on the other hand, showed clean chromatograms. For example, Figure 2B shows a fraction taken after 30 min of ROL-catalyzed reaction in DIPE. For simplicity, ROL-catalyzed esterification in DIPE was chosen as the preferred reaction system, since this was also used in the optimized methanolysis reaction. The time course for the ROL-catalyzed esterification of 2-monoricinolein with ricinoleic acid in DIPE is shown in Figure 5. As can be seen from the figure, the reaction is rapid, with a maximum yield of 58% 1,2(2,3)-diricinolein after one hour of reaction. Reactions longer than one hour gave decreased amounts of diricinolein in the reaction mixture.

Figure 6 shows an HPLC chromatogram of the collected 1,2(2,3)-diricinolein peak, indicating the purity of the final product. Furthermore, ^{1}H and ^{13}C NMR showed that the purity of 1,2(2,3)-diricinolein vs. possibly coeluting 1,3-diricinolein was 97.2%.

In conclusion, ROL was the optimal lipase for catalyzing both the conversion of triricinolein to 2-monoricinolein and the esterification reaction of 2-monoricinolein with ricinoleic acid. DIPE was chosen as the best reaction solvent at a_w of 0.11. Schmid *et al.* (14) also found ROL to be the most suitable for

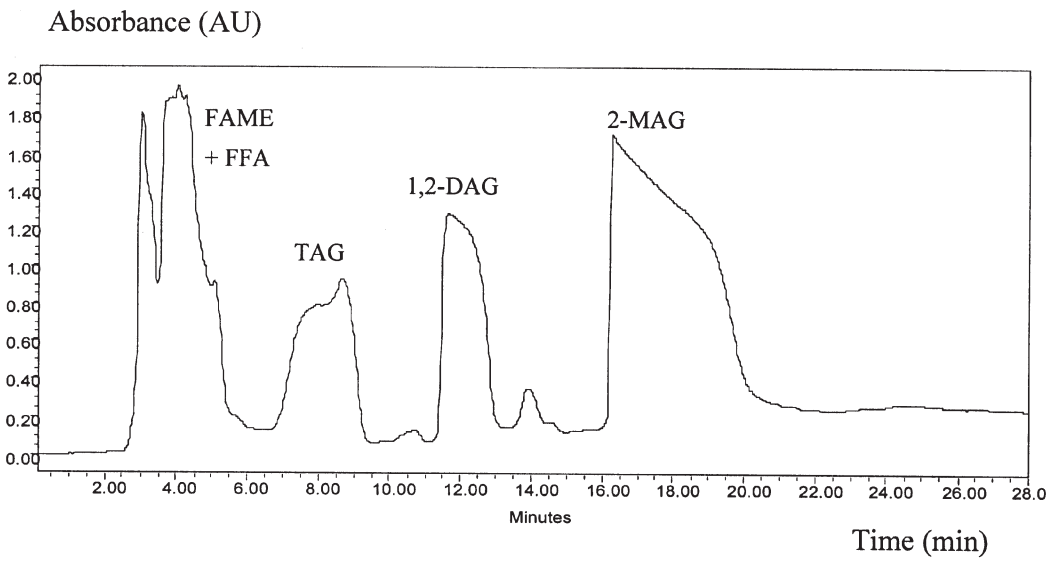

FIG. 4. Preparative HPLC of the reaction products after 30 min of ROL-catalyzed methanolysis reaction of triricinolein in DIPE. FAME, methyl ricinoleate; FFA, ricinoleic acid; for other abbreviations see Figure 2.

selective synthesis of 1,3-oleoyl-2 palmitoyl-glycerol. The optimal reaction time was only 30 min for the methanolysis reaction and one hour for the esterification reaction. For the methanolysis reaction, these conditions gave a 2-monoricinolein yield of 78% and a purity of nearly 100%. For the second esterification reaction, the optimized reaction gave a 1,2(2,3)-diricinolein yield of 58% and a purity of 97.2%. This reaction scheme can be used for the production of high-purity (>97%) radiolabeled 1,2(2,3)-diricinolein. Work is in progress in our laboratory to make this radiolabeled DAG and to test it in reactions with the DGAT enzyme expressed in yeast.

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TABLE 2
Maximum Yields of 1,2(2,3)-Diricinolein (%) from Lipase-Catalyzed Esterification of Ricinoleic Acid with 2-Monoricinolein[a]

Lipase	Solvent	
	DIPE	Toluene
CALB	7 (0.5 h)	15 (1 h)
RML	54 (1.5 h)	56 (1 h)
ROL	58 (1 h)	58 (0.5 h)
TLL	43 (1.5 h)	59 (1.5 h)
ANL	27 (48 h)	32 (48 h)
CRL	9 (48 h)	18 (48 h)
PRL	6 (48 h)	6 (48 h)
PCL	43 (24 h)	43 (24 h)
PFL	35 (24 h)	57 (48 h)

[a]Data points represent the means of two experiments. The reaction times giving these maximum recoveries are shown within parentheses. CRL, *Candida rugosa* lipase; PRL, *Penicillium roquefortii* lipase; PCL, *Pseudomonas cepacia* lipase; PFL, *Pseudomonas fluorescence* lipase; for other abbreviations see Table 1.

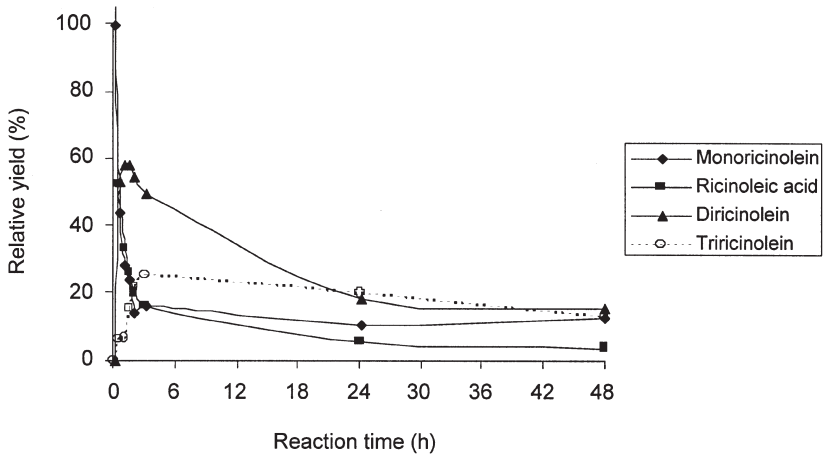

FIG. 5. Time course for the ROL-catalyzed esterification reaction between 2-monoricinolein and ricinoleic acid in DIPE. Data points represent the means of two experiments. For abbreviations see Figure 2.

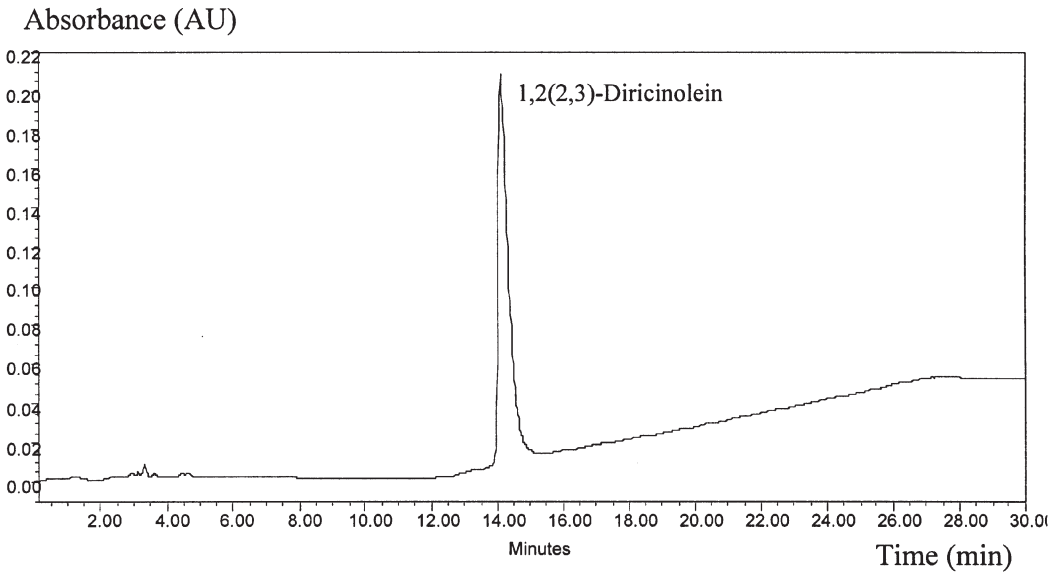

FIG. 6. HPLC of synthesized 1,2(2,3)-diricinolein.

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A Significant Inverse Relationship Between Concentrations of Plasma Homocysteine and Phospholipid Docosahexaenoic Acid in Healthy Male Subjects

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ABSTRACT: The aim of this study was to investigate the possibility of a relationship between plasma homocysteine (Hcy) and phospholipid FA (PUFA) in healthy Australian males. One hundred thirty six healthy male subjects aged 20–55 yr were recruited from the Melbourne metropolitan area. Each volunteer completed a semiquantitative food frequency questionnaire and gave a blood sample. Plasma Hcy concentrations were determined by an established HPLC method; the plasma phospholipid FA were determined by standard methods. Plasma Hcy concentration was significantly negatively correlated with plasma phospholipid concentration of the PUFA 20:5n-3 (r = –0.226, P = 0.009), 22:5n-3 (r = –0.182, P = 0.036), 22:6n-3 (r = –0.286, P = 0.001), total n-3 (r = –0.270, P = 0.002) and the ratio n-3/n-6 PUFA (r = –0.265, P = 0.002), and significantly positively correlated with 20:4n-6 (r = 0.180, P = 0.037). In the partial correlation analysis, after controlling for serum vitamin B_{12} and folate concentration, plasma Hcy was significantly negatively correlated with the plasma phospholipid concentration of 22:6n-3 (r = –0.205, P = 0.019), total n-3 (r = –0.182, P = 0.038) and the ratio n-3/n-6 PUFA (r = –0.174, P = 0.048). Evidence indicates that an increased concentration of n-3 PUFA in tissues has a beneficial effect on cardiovascular health. Our findings provide further evidence that increased consumption of dietary n-3 PUFA increases the concentration of n-3 PUFA in plasma phospholipid, which is associated with a protective effect on cardiovascular diseases and lower plasma Hcy levels. The mechanism that might explain the association between plasma 22:6n-3 and Hcy levels is not clear.

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Increased plasma homocysteine (Hcy) levels have been suggested to be an independent risk factor for cardiovascular diseases (1–3). Hcy is an intermediate metabolite in the metabolism of methionine to cysteine. The normal metabolism of Hcy involves two pathways: remethylation and transsulfuration. Remethylation of Hcy to methionine requires vitamin B_{12} (methylcobalamin form) as a coenzyme for Hcy methyltransferase (methionine synthetase) and N^5-methyl-tetrahydrofolate as a methyl donor. The transsulfuration pathway of Hcy to cysteine requires vitamin B_6 as a coenzyme for both cystathionine β-synthase (converts Hcy to cystathionine) and cystathionine lyase (converts cystathionine to cysteine). A lack of dietary vitamin B_{12} and/or folic acid or vitamin B_6 results in elevation of plasma Hcy (4). We previously reported that plasma Hcy concentration was significantly negatively correlated with serum vitamin B_{12} concentration in healthy Australian males, but there was no significant correlation with folate (5).

Increased dietary intake of n-3 PUFA will lead to an increased tissue level of these FA, which has a beneficial effect on cardiovascular morbidity and mortality (6,7) *via* reduction in blood pressure (8) and serum/plasma TAG levels (9,10), antithrombotic effect (11,12), anti-inflammatory effect (13), increase in heart rate variability (14), and secondary prevention of cardiovascular disease (15,16).

Grundt *et al.* (17) have reported a beneficial effect after 1 yr of a high-dose n-3 PUFA (2 gelatin capsules daily, each capsule containing 850–882 mg EPA and DHA ethylesters) treatment (n = 150) on lowering plasma Hcy levels in a prospective, randomized, double-blind study compared with corn oil (n = 150) in acute myocardial infarction (MI) patients (P = 0.022) (17). However, it is not clear what the mechanism for the finding is. We had an opportunity to study whether plasma phospholipid (PL) PUFA concentrations, as surrogate markers of dietary intake of PUFA, are correlated with Hcy, since we had collected semiquantitative food frequency questionnaires and blood samples from four groups of men with widely varying intakes of n-3 PUFA. The aim of this study was to investigate the relationship of plasma Hcy with PL FA concentrations (as biomarker) in healthy Australian male subjects with variable n-3 PUFA intake.

METHODS

Subjects. The present study formed a part of a larger project investigating the association between diet and cardiovascular risk factors in Australian men (18). The study protocol was approved by the Human Ethics Committee of the RMIT University (Melbourne, Australia), and all subjects were volunteers who gave their written consent prior to participation in the study. A total of 139 subjects (18 vegans; 43 ovolacto vegetarians; 60 omnivores; 18 high meat-eaters ≥ 280 g raw meat/d)

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Abbreviations: Hcy, homocysteine; MI, myocardial infarction; PL, phospholipid

were recruited from the Melbourne metropolitan area; all were healthy nonsmoking males in the age range 22–55 yr, with no family history of cardiovascular disease, and not taking any vitamin-fortified supplements.

Blood collection and dietary analysis. All subjects attended the RMIT University Medical Clinic on one morning following a 12-h overnight fast. Blood collection and dietary analysis were as described previously (18).

Laboratory measurements. Total Hcy was quantified in plasma as the ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate derivative (19), using a HPLC separation, fluorometric detection method as described by Dudman *et al.* (20). To determine plasma PL FA, the total lipid of plasma was extracted with solvents, the plasma PL fraction was separated by TLC, and the methyl esters of the FA of the plasma PL fraction were prepared and separated by GLC as described previously (18).

Statistics. The data analyses were performed using a SPSS version 12 (SPSS Inc., Chicago, IL) software program. The correlation between Hcy and plasma PL FA concentration was initially determined by bivariate analysis. Partial correlation analysis was further performed, controlled for serum concentrations of vitamin B_{12} and folate. The level of significance was $P < 0.05$.

RESULTS

The original data on fish intake, plasma PL concentrations of various n-3 PUFA, and arachidonic acid have been published previously (18). The mean intake of fish was 28, 26, 1.4, and 0.7 g/d in high meat-eaters, moderate meat-eaters, ovolacto vegetarians, and vegans, respectively. Plasma PL concentrations of mean total n-3 PUFA were 7.2, 6.9, 4.5, and 4.0 mg/100 mL, respectively. The plasma PL 22:6n-3 concentrations were 4.1, 4.0, 2.3, and 2.1, respectively, and the 20:5n-3 concentrations were 1.4, 1.2, 0.8, and 0.6, respectively. The plasma PL concentrations of 20:4n-6 were 12.8, 12.6, 10.0, and 10.6 mg/100 mL, respectively.

Plasma Hcy concentrations were greater than 20 μmol/L in 5.9% of the subjects, between 20 and 15 μmol/L in 16.2%, between 14.9 and 10 μmol/L in 52.9%, and less than 10 μmol/L in 25%. Table 1 shows the correlations between the concentrations of plasma Hcy and PL FA. Plasma Hcy concentration was significantly negatively correlated with plasma PL concentration (mg/100 mL) of 20:5n-3 ($r = –0.226$, $P = 0.009$), 22:5n-3 ($r = –0.182$, $P = 0.036$), 22:6n-3 ($r = –0.286$, $P = 0.001$), total n-3 ($r = –0.270$, $P = 0.002$) and the ratio n-3/n-6 PUFA ($r = –0.265$, $P = 0.002$), and significantly positively correlated with 20:4n-6 ($r = 0.180$, $P = 0.037$).

TABLE 1
Bivariate Correlations Between Plasma Concentrations of Homocysteine and Phospholipid FA[a]

FA	Std. coeff.	*P*-value
20:4n-6	0.18	0.037
20:5n-3	–0.226	0.009
22:5n-3	–0.182	0.036
22:6n-3	–0.286	0.001
Total n-3	–0.27	0.002
n-3/n-6	–0.265	0.002

[a]n = 136; Std. coeff. = standardized coefficients.

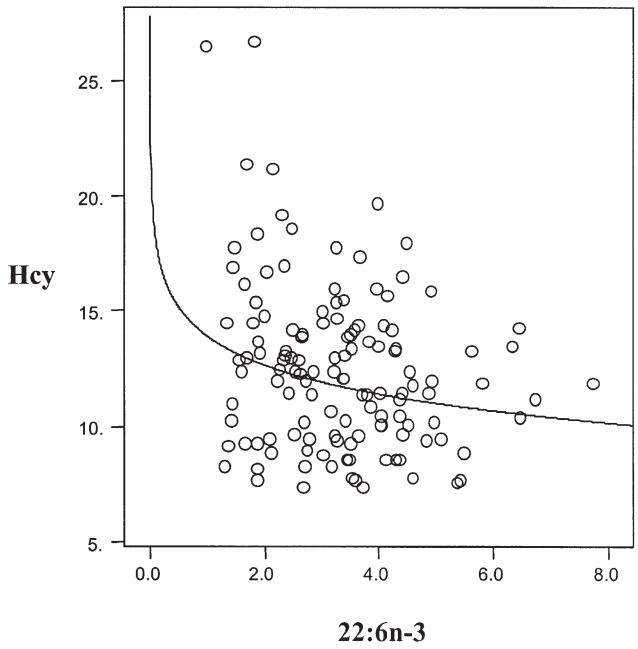

FIG. 1. Correlation of plasma concentrations of homocysteine (Hcy) with phospholipid DHA concentration.

In the partial correlation analysis, after controlling for dietary groups and serum concentrations of vitamin B_{12} (pg/mL) and folate (ng/mL), plasma Hcy was significantly negatively

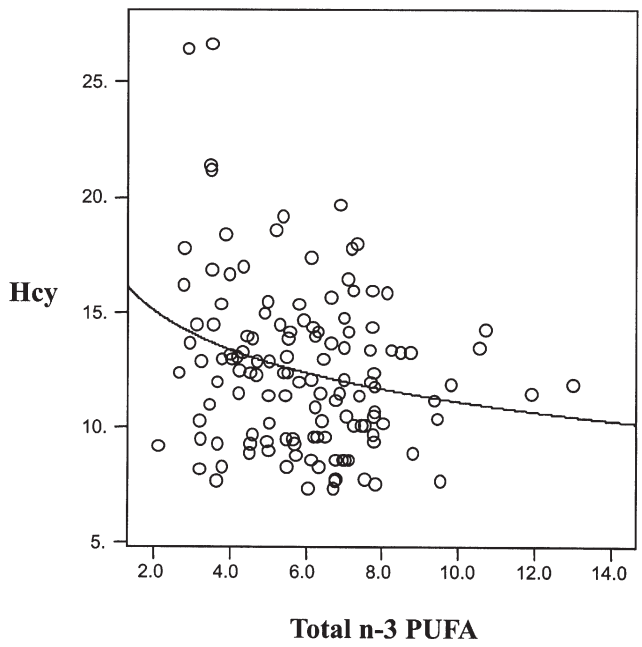

FIG. 2. Correlation of plasma concentrations of Hcy with phospholipid total n-3 PUFA concentration. For abbreviation see Figure 1.

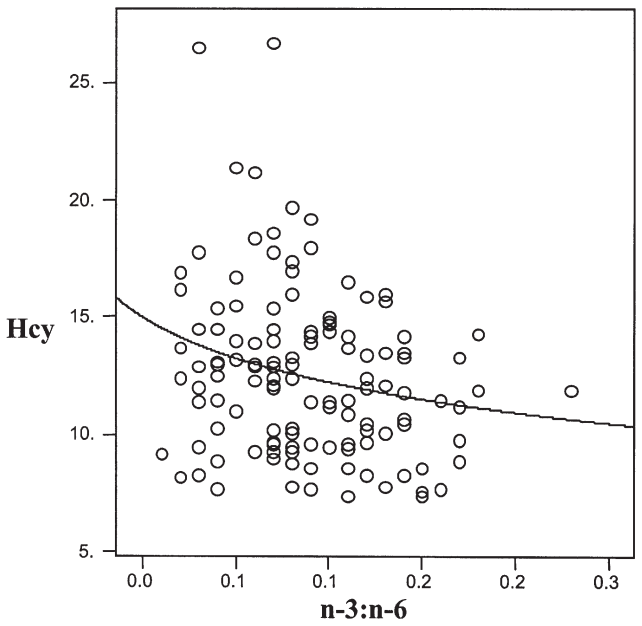

FIG. 3. Correlation of plasma Hcy concentration with phospholipids ratio of n-3 to n-6 PUFA. For abbreviation see Figure 1.

correlated with plasma PL concentration of 22:6n-3 (r = –0.205, P = 0.019), total n-3 (r = –0.182, P = 0.038), and the ratio n-3/n-6 PUFA (r = –0.174, P = 0.048) (Figs. 1–3, Table 2).

Table 3 shows that the plasma PL 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3, total n-3 PUFA, and n-3/n-6 ratio were significantly positively correlated with serum vitamin B_{12}, but not folate, in the present study.

DISCUSSION

Results from epidemiological, case-control, and cross-sectional studies have identified that elevated concentrations of plasma Hcy are an independent risk factor for cardiovascular disease. In a large prospective study from the United States (Physician's Health Study) (1), 14,916 male physicians, aged 40–84 yr, with no prior MI or stroke, provided plasma samples at baseline and were followed up for 5 yr. The results indicated that the 271 subjects who later had MI had significantly higher mean baseline levels of Hcy than subjects that were matched with paired controls who remained free of infarction. Subjects whose Hcy levels were in the highest 5% had about three times the risk of MI compared with those with lower levels (P = 0.005), even after adjustment for a variety of coronary risk factors. In a large-scale prospective nested case control study (Tromsø Study) (2), 21,826 subjects (10,963 males and 10,863 females), aged 12–61 yr, who were free from MI at the screening were studied. Three years later, 123 subjects developed coronary heart disease. Levels of serum Hcy were found to be higher in cases than in controls (P = 0.002), but with no threshold level. In a cross-sectional study (Framingham Heart Study) of 1,041 elderly subjects (418 men and 623 women; aged 67–96 yr) (21), the results showed a clearly graded increase in the prevalence of carotid artery stenosis with increasing plasma levels of Hcy. Individuals in the group having the highest levels of Hcy had twice the risk of severe stenosis when compared with the group with lowest Hcy levels. Similar findings were reported for the risk of carotid artery thickening (22), for angiographically defined coronary artery stenosis (23), for occlusive arterial disease (24), for MI (22,25), and for stroke (26). A study in 482 patients already at high risk of atherosclerosis from hyperlipidemia, assessed by logistic regression, indicated that high Hcy levels were an independent risk factor for atherosclerotic vascular disease. The relative risk for atherosclerotic events was almost three times higher (P = 0.0004) in patients in the top (≥11.4 μmol/L) compared with the bottom (<6.9 μmol/L) quintile of Hcy. There were significant Hcy/HDL (P = 0.012) and Hcy/TAG (P = 0.02) interaction terms. The highest risk of an atherosclerotic event was seen when Hcy was high and HDL and TAG were low (27). Reis *et al.* (28) investigated the correlation between the Hcy and early cerebrovascular disease in 33 (19 male, 14 female) patients under 55 yr old who suffered a stroke from 3 mon to 1 yr before the study. The patients were

TABLE 2
Partial Correlations Between Plasma Concentrations of Homocysteine and Phospholipid FA, Controlled for Confounding Factors[a]

	After controlling for serum vitamin B_{12} and folate	
FA	Std. coeff.	*P*-value
22:6n-3	–0.205	0.019
Total n-3	–0.182	0.038
n-3/n-6	–0.174	0.048

[a]For footnote see Table 1.

TABLE 3
Bivariate Correlations Between Serum Vitamin B_{12} and Folate and Plasma Phospholipid FA[a]

	Serum vitamin B_{12}		Serum folate	
FA	Std. coeff.	*P*-value	Std. coeff.	*P*-value
20:4n-6	0.228	<0.0001	0.065	0.452
20:5n-3	0.340	<0.0001	0.042	0.633
22:5n-3	0.331	<0.0001	0.025	0.776
22:6n-3	0.364	<0.0001	–0.124	0.154
Total n-3	0.379	<0.0001	–0.128	0.141
n-3/n-6	0.396	<0.0001	–0.166	0.055

[a]For footnote see Table 1.

matched with a group of normal subjects in terms of age and sex. They found that hyperhomocysteinemia was a risk factor for thrombotic cerebrovascular disease before the age of 55 (28). In a nested case-control study of 5,661 British men aged 40–59 yr during a12-yr followup, there were 141 incidents of stroke among men with no history of stroke at screening. Serum Hcy concentrations were significantly higher in 107 cases than 118 controls (who did not develop a stroke or MI during followup) ($P = 0.004$) (29).

Hcy is an intermediate metabolite of the metabolism of methionine to cysteine. Normal metabolism from Hcy to cysteine involves two pathways: remethylation and transsulfuration, which require vitamins B_{12}, B_6, and folate as coenzymes. Lack of vitamins B_{12} and/or folic acid or vitamin B_6 will results in elevation of plasma Hcy (4). Previously, we reported a significant negative correlation between plasma Hcy concentration and serum vitamin B_{12} concentration, and we also found no significant correlation with plasma folate in healthy male Australians (5). A similar finding has been reported from Chile (30).

In a double-blind intervention study, 57 healthy subjects were randomly divided to receive either a daily dose of 4 g of 85% 20:5n-3/22:6n-3 ($n = 28$) or corn oil ($n = 29$) for 12 wk. No significant changes in plasma Hcy levels appeared in either group after 12 wk of treatment compared with baseline (31). However, following 1 yr of n-3 PUFA treatment in the acute MI patients from the same research group with the same dose of n-3 PUFA, plasma Hcy levels were significantly lower in the n-3 PUFA group ($n = 150$) than in the corn oil group ($n = 150$) at follow-up ($P = 0.022$) (17). The authors attributed these contradictory results to the limited subject numbers in their first study (31). Bohles *et al.* (32) determined the plasma Hcy and erythrocyte PL FA in mothers ($n = 60$, age range 21–39 yr) and their newborn children. Maternal plasma Hcy concentration was significantly positively correlated with that of their newborns ($r = 0.71$, $P < 0.0001$). The maternal plasma Hcy levels were significantly negatively correlated with their offspring erythrocyte PL 22:6n-3 concentrations ($r = -0.51$, $P < 0.0003$) (32). However, there are no data in the literature on the influence of habitual n-3 PUFA intake on plasma Hcy levels.

Results from the present study showed that plasma Hcy concentration was significantly negatively correlated with the concentrations of plasma PL 20:5n-3, 22:5n-3, 22:6n-3, total n-3 PUFA, and ratio of n-3 to n-6 PUFA, and significantly positively correlated with 20:4n-6. It has been known that both n-3 PUFA and vitamin B_{12} only occur in animal- and marine-based food. In fact, plasma PL 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3, total n-3 PUFA, and n-3:n-6 ratio were significantly positively correlated with serum vitamin B_{12}, but not folate in the present study (Table 3). However, after controlling for serum vitamin B_{12} and folate, plasma Hcy concentration was still significantly negatively correlated with the concentrations of plasma PL 22:6n-3, total n-3 PUFA, and ratio of n-3 to n-6 PUFA. A possible mechanism for this relationship is that n-3 PUFA, especially 22:6n-3, may modulate gene expression of an enzyme(s) that are involved in formation and metabolism of plasma Hcy.

In conclusion, the present results provide further information that increased n-3 PUFA levels in plasma PL may have a protective effect on cardiovascular diseases in relation to lowering plasma Hcy levels. The mechanism that might explain the association between plasma DHA and Hcy levels is not clear.

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The Real Radical Generator Other Than Main-Product Hydroperoxide in Lipid Autoxidation

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ABSTRACT: The theory of initiation in lipid autoxidation, which deals with the supply of radicals to the chain reaction, has not been substantively advanced for several decades. Most researchers have long assumed a mechanism of initiation in which main-product hydroperoxide is centrally responsible for autocatalytic radical generation. However, this paper, in which we investigate autoxidizing methyl linoleate, presents decisive evidence against such an assumption: Autoxidation-accelerating activity under mild conditions was not found in the chromatographically separated main-product hydroperoxide fraction but was found in other fractions; and highly active substances with structures containing a peroxide-linked dimer with two hydroperoxy groups were actually obtained.

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A great deal of attention has been focused recently on the damage to biological systems by free radicals. An important source of free radicals is the autoxidation of lipids. Despite the large number of studies conducted in this field, the theory of initiation, which deals with the supply of radicals to the autoxidation chain reaction, has not been substantively advanced for several decades as can be seen in comparing reviews (1–3). The factors that govern the rate of oxidation and the level of radicals remain incompletely elucidated. The main product of autoxidation is a type of hydroperoxide. Since the 1940s, most researchers have assumed a mechanism of initiation in which this hydroperoxide is centrally responsible for autocatalytic radical generation, but we have entertained a minority opinion (4–6) that this role is not in fact played by the hydroperoxide. This paper, in which we investigate autoxidizing methyl linoleate (oxML), presents decisive evidence corroborating our opinion.

EXPERIMENTAL PROCEDURES

Linoleate materials. Methyl linoleate and pentyl linoleate were prepared as described (7). Methyl linoleate was also supplied from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). In a preliminary experiment, the metal contents of prepared methyl linoleate were determined and found to be 0.17 ppm Fe and 0.053 ppm Cu before vacuum distillation and 0.011 ppm Fe and 0.0033 ppm Cu after six repetitions of vacuum distillation. The distilled methyl linoleate, however, did not exhibit a lower rate of autoxidation; once-distilled methyl linoleate and the purchased methyl linoleate showed oxidation tendencies that were not different from the material that had been redistilled six times. So, once-distilled methyl linoleate and the purchased material were used in these experiments. For the measurement of oxidation rate described herein, pentyl linoleate was purified by passage through a silica gel column using the purified solvents described later.

HPLC fractionation of oxML. A sample of oxML at 20°C, containing about 2 μmol main-product hydroperoxide, was subjected to HPLC on a 7.6 × 250 mm Nucleosil®100–5 column (Agilent, Palo Alto, CA) using hexane/diethyl ether (92:8, vol/vol) at a flow rate of 4 mL/min, monitored at 234 nm. Main-product hydroperoxide (MLOOH) was eluted from 11 to 20 min and the solvent was exchanged with methanol. Eluates before and after the elution of MLOOH were combined (oxML-MLOOH). Fractions obtained were concentrated under reduced pressure below 30°C.

Measurement of autoxidation-accelerating activity. Two microliters of pentyl linoleate, the oxidation substrate, was placed in a flat-bottomed vial of 9 mm i.d. The sample to be tested was added in acetone (0.2–0.4 mL), and the acetone was removed under a stream of nitrogen. The vial was allowed to stand in the dark at 20°C for 12 h, at 35°C for 3.5 h, or at 50°C for 1 h. The contents of the vial were then analyzed by HPLC for main-product hydroperoxide of pentyl linoleate, using a 4.6 × 150 mm Nucleosil 100–5 column with hexane/diethyl ether (88:12, vol/vol) at a flow rate of 1.5 mL/min, monitored at 234 nm (7). The oxidation rate was expressed using the rate of formation of main-product hydroperoxide. Each figure described and used for the subsequent calculation was the average obtained from 4–6 runs. The autoxidation-accelerating activity was calculated on the basis that the square of the oxidation rate increases in linear fashion with the concentration of oxidation-accelerating (radical-generating) material added, and was expressed as 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) equivalent (Azo eq).

Purification of solvents. Distilled hexane, diethyl ether, and methanol were used for HPLC fractionation. A glass apparatus with a rectifying column was used. Methanol was distilled under reduced pressure. The solvents were tested for acceptability as follows: 60 mL of solvent obtained from distillation was placed in a flask and evaporated under reduced pressure.

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Abbreviations: Azo eq, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) equivalent; MLOOH, main-product hydroperoxide of methyl linoleate; oxML, autoxidizing methyl linoleate.

The flask was washed with 0.2–0.4 mL acetone, which was added to 2 μL pentyl linoleate, and the oxidation rate of the pentyl linoleate was measured. An unchanged rate showed that the solvent was acceptable for use.

Sephadex LH-20 column chromatography. A sample of less than 1 g was chromatographed on a 12.5 × 1100 mm Sephadex LH-20 column (Amersham Biosciences, Uppsala, Sweden) using methanol at a flow rate of about 0.5 mL/min. The eluates were examined by TLC.

Reduction with triphenylphosphine. An excess of 2% triphenylphosphine in hexane was added to the sample to be reduced. After 30 min, the mixture was separated in a 7.6 × 250 mm Nucleosil 100–5 column using a hexane/2-propanol solvent (97:3) at a flow rate of 4 mL/min, and the reduced material was recovered.

Reduction with $NaBH_4$. An excess of 2% $NaBH_4$ in methanol containing 0.05 M sodium methoxide was added to the sample to be reduced. After 30 min, a drop of acetic acid was added. The sample was diluted with water and added to a 7.6 × 250 mm Inertsil column (GL Science, Tokyo, Japan) monitored at 234 nm, and the reduced material was recovered with methanol at a flow rate of 2 mL/min. Methyl hydroxyoctadecadienoate was eluted separately from the unreacted materials.

GC–MS. After conversion to the trimethylsilyl derivative, the sample was analyzed by GC–MS (JEOL JMS600) with a DB-1 capillary column (length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μm; J&W Scientific, Folsom, CA) at a flow rate of 1 mL/min from 150 to 300°C at a ramping rate of 8°C/min. Methyl trihydroxyoctadecenoate was identified by referring to previously reported data (8).

Determination of moderately reducible peroxide. A sample containing about 0.2 μmol of conjugated diene was refluxed in 1.2 mL of 2-propanol containing 0.12 mL acetic acid and 100 mg NaI for 20 min (9). The mixture was then titrated with 0.005 mol/L $Na_2S_2O_3$.

RESULTS

We estimated the autoxidation-accelerating activity of oxML and fractions separated from it (Table 1). In experiment 1, the activity of chromatographically separated main-product hy-

TABLE 1
Autoxidation-Accelerating Activity of Autoxidizing Methyl Linoleate and Separated Fractions[a]

	Oxidation temperature					
	20°C		35°C		50°C	
	Ox rate[b] μmol/mL·h	Azo eq[c] 10^{-5} M	Ox rate[b] μmol/mL·h	Azo eq[c] 10^{-5} M	Ox rate[b] μmol/mL·h	Azo eq[c] 10^{-5} M
Experiment 1[d]						
Control[e]	0.72 ± 0.01					
oxML1[f]	1.20 ± 0.22	6.03				
MLOOH[g] from oxML1[f]	0.24 ± 0.02	−2.65				
oxML2[f]	1.27 ± 0.17	7.44				
MLOOH[g] from oxML2[f]	0.39 ± 0.06	−2.17				
oxML3[f]	1.98 ± 0.18	23.6				
MLOOH[g] from oxML3[f]	0.39 ± 0.13	−2.00				
Fraction X[h]	5.08 ± 0.81	183				
Experiment 2[i]						
Control[e]	0.79 ± 0.36		4.76 ± 1.71		6.63 ± 0.66	
oxML4[f]	2.22 ± 0.31	29.1	8.61 ± 1.59	10.9	25.6 ± 3.8	9.46
MLOOH[g]	0.81 ± 0.40	0.32	4.93 ± 1.12	0.51	13.7 ± 2.1	2.47
oxML–MLOOH[j]	2.05 ± 0.23	24.2	10.0 ± 0.94	16.4	27.9 ± 0.23	11.1

[a]See Experimental Procedures section for experimental details.
[b]The oxidation rate of pentyl linoleate was represented by the formation rate of its main-product hydroperoxide.
[c]Autoxidation-accelerating activity was calculated from the oxidation rate of pentyl linoleate and expressed by the equivalent concentration of 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile).
[d]In Experiment 1, 2 μL of pentyl linoleate, the oxidation substrate, was placed in a vial with a sample to be tested for autoxidation-accelerating activity. Each sample contained 234 nm-absorbing material of 1.5 OD × mL, corresponding to about 5.5×10^{-8} mol hydroperoxide.
[e]Control: the oxidation substrate only.
[f]oxML1, oxML2, oxML3, oxML4: autoxidizing methyl linoleate; main-product hydroperoxide contents of oxML1, oxML2, oxML3, and oxML4 were about 30, 250, 500, and 600 μmol/mL, respectively.
[g]MLOOH: main-product hydroperoxide fraction chromatographically separated from autoxidizing methyl linoleate.
[h]Fraction X: a polar fraction from autoxidizing methyl linoleate as shown in Figure 1.
[i]In Experiment 2, 2 μL of pentyl linoleate was used in each vial; oxML4 of 1.5 OD × mL was placed in a vial; for MLOOH and oxML–MLOOH samples, the amount chromatographically fractionated from oxML4 to give 1.5 OD × mL was placed in a vial.
[j]oxML–MLOOH: combined sample of residual fractions, namely, chromatographically separated fractions other than MLOOH.

droperoxide was compared with that of unseparated oxML, with each sample containing materials which had the same 234-nm absorbing ability as represented by the same OD × mL value. The activity of the unseparated linoleate increased as autoxidation proceeded, but no activity was exhibited at 20°C by main-product hydroperoxide obtained at any stage. In experiment 2, a chromatographically separated main-product hydroperoxide fraction and a combined sample of residual fractions were examined at 20, 35, and 50°C. The hydroperoxide fraction was not active at 20 or 35°C, and the combined sample showed almost the same activity as the unseparated sample. At 50°C, some activity was observed in the main-product hydroperoxide fraction, although whether the activity was intrinsic to the hydroperoxide was uncertain.

Autoxidation-accelerating activity in oxML was distributed widely over fractions separated by normal and reversed-phase HPLC. A highly active fraction, X, separated by normal-phase HPLC, was found to be more polar than main-product hydroperoxide (Fig. 1) and to have a UV absorption maximum near 234 nm and an activity of 183×10^{-5} M Azo eq at 20°C, as shown in experiment 1 of Table 1. The concentration of X rapidly increased at a later stage of autoxidation. Its amount relative to that of main-product hydroperoxide on the absorption basis at 234 nm was 2.1% at a hydroperoxide level of 380 μmol/mL, and 5.6% at 700 μmol/mL. If we assume that X is present in oxML3 (a highly autoxidizing sample in Table 1) at roughly a 3% level on an absorption basis, one can estimate from the activity data in Table 1 that X is responsible for about one-fourth of the total activity of oxML3.

Although X was a mixture, the components were similar to each other in various behaviors. Using a Sephadex LH-20 column with methanol, the fraction was eluted from 100 to 110 mL while the main-product hydroperoxide eluted from 130 to 140 mL. In the 100–110 mL region, both DAG prepared from sunflower oil and a nonpolar dimer of methyl linoleate (10) were eluted, which suggested that X had a dimeric structure. X contained easily reducible peroxide, which was reduced with iodide at room temperature. Triphenylphosphine also could reduce it as follows:

$$\text{ROOH} \xrightarrow{\Phi_3P} \text{ROH}$$

The reduction product with triphenylphosphine, Y, was found in the dimer region of the Sephadex column eluates (100–110 mL). On a molar basis, the amount of triphenylphosphine consumed was twice the amount of conjugated diene found by measuring absorption at 234 nm, which suggests that the compounds of group X may be characterized as dimers with two hydroperoxy groups. On treatment with $NaBH_4$, Y gave methyl hydroxyoctadecadienoate and methyl trihydroxyoctadecenoate. The former is the same compound as the reduced derivative of main-product hydroperoxide. The latter gave four peaks at retention times of 15.50, 15.78, 15.92, and 15.98 min in GC–MS. Their mass spectra had fragment ions at *m/z* 545 (M − 15), 460 (M − 100), 387, 301, 259, 173, and 155, which are characteristic of methyl 9,10,13-trihydroxy-11- and 9,12,13-trihydroxy-10-octadecenoate. The intensity ratios of *m/z* 259 and 173 showed that the first and third peaks were methyl 9,10,13-trihydroxy-11-octadecenoate and the second and the fourth peaks were methyl 9,12,13-trihydroxy-10-octadecenoate (8). The cleaved bond was confirmed to be a moderately reducible peroxide since it was reduced with iodide by refluxing in 2-propanol. So, the above reduction with $NaBH_4$ can be written as:

$$\text{ROOR}' \xrightarrow{NaBH_4,\ H^+} \text{ROH} + \text{R'OH}$$

Although Y contained unreacted materials after treatment with $NaBH_4$, they were not found in the first part of the eluate from a 7.6 × 250 mm Nucleosil 100–5 column using a hexane/2-propanol solvent (97:3) at a flow rate of 4 mL/min, which was analyzed for peroxide. Its peroxide and conjugated diene contents were found to be roughly equal on a molar basis, indicating that Y contained a dimer in which two moieties (methyl octadecadienoate and methyl dihydroxyoctadecenoate) are linked with a peroxide bridge.

It was difficult to isolate such a fraction chromatographically from X, as $NaBH_4$ treatment of this fraction resulted in changed materials only. So, X was divided into fractions giving different yields of methyl hydroxyoctadecadienoate on a 7.6 × 250 mm Nucleosil®100–5 column using a hexane/2-propanol solvent (99:1) at a flow rate of 4 mL/min. With each sample containing 2.0 OD × mL of material, three fractions giving methyl hydroxyoctadecadienoate (at 19.1, 25.7, and 38.8% yields; from total 234 nm absorption) showed autoxidation-accelerating activities of 70.3, 92.2, and 141×10^{-5} M Azo eq, respectively, demonstrating a clear proportional relationship between methyl hydroxyoctadecadienoate yield and activity. The correlation coefficient of 0.997 indicated that the substances that generated methyl hydroxyoctadecadienoate were themselves autocatalysts.

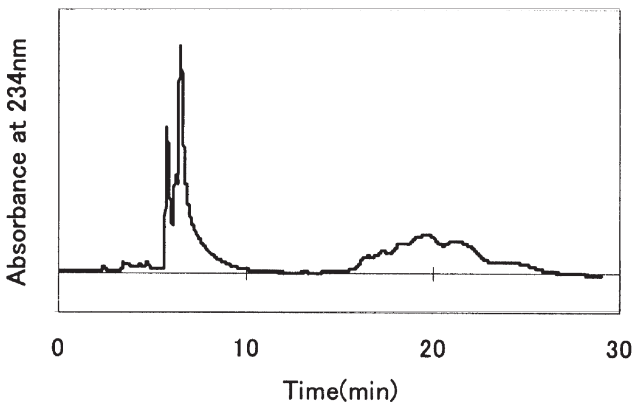


FIG. 1. Elution pattern of active fraction X on HPLC. On a 7.6 × 250 mm Nucleosil®100–5 column (Agilent, Palo Alto, CA) using hexane/2-propanol (99:1, vol/vol) at a flow rate of 4 mL/min, monitored at 234 nm, fraction X was eluted during the 16–26 min period and the main-product hydroperoxide was eluted in the 6–8 min period.

DISCUSSION

The results in Table 1 show that main-product hydroperoxide has little autocatalytic radical-generating activity at room or body temperature, and that autocatalytic roles are played by other substances. It seems extremely important that the mild conditions used in the present experiments cover a very wide area of lipid autoxidation in biological systems and food storage.

As ordinary hydroperoxide does not tend to generate radicals by homolytic cleavage, there has long been speculation that main-product hydroperoxide may generate radicals in the presence of trace transition metals (2). However, if main-product hydroperoxide acted as the main autocatalyst with trace metals that still remained in the samples of our experiments and the separated hydroperoxide became inactive by removal of the metals, it would seem impossible to explain why the combined sample of the residual parts retained almost all the activity of the unseparated sample.

It is worth asking why we had not previously been able to reach the conclusion obtained here. Our answer is that there was considerable difficulty in finding suitable methods for obtaining these results. For chromatographic separation, it was necessary to use solvents that had absolutely no effect on the subsequent oxidation experiment; also, to obtain an inactive main-product hydroperoxide fraction, the sample that was applied to HPLC had to be limited to a small size.

For the autocatalytic substances in fraction X, an isomeric mixture of dimer structures is likely, which has reported by Miyashita *et al.* (11) in oxidized products of the main-product hydroperoxide of methyl linoleate; the probable mechanism is the addition of a peroxy radical to the conjugated diene of main-product hydroperoxide followed by the usual propagation process, as shown in Scheme 1.

Such formation of a peroxy bridge is known to occur in the autoxidation of conjugated olefins, giving relatively unstable olefin-oxygen copolymers; and their instability is explained in terms of concerted decomposition of their repeated α,β-diperoxide structures as shown in Scheme 2 (12,13). The formation of aldehydes is thought to be energetically favorable for the reaction. The reaction is estimated using group additivity rules (14) to be exothermic at levels of about 18 kcal/mol for alkanal formation and 22 kcal/mol for conjugated alkenal formation. It is noted that radicals and aldehydes are simultaneously formed on decomposition.

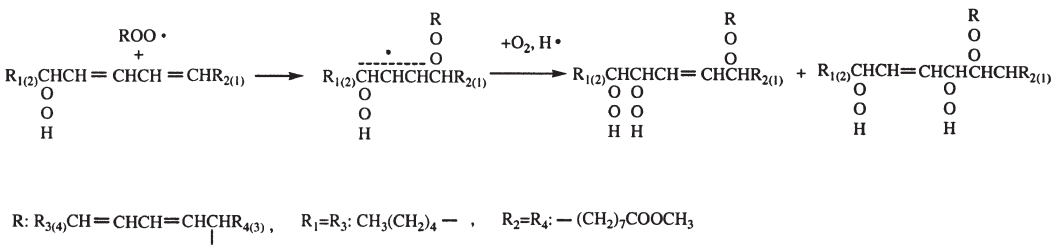
As the present substances have a minimum repetition structure of the polymer, they are expected to display considerable radical-generating activity.

Although present substances are only a kind of the autocatalysts in oxML and many other types may exist, it seems very important that a kind of potent autocatalysts was actually obtained here and we got a promising clue to the elucidation of the autocatalytic initiation.

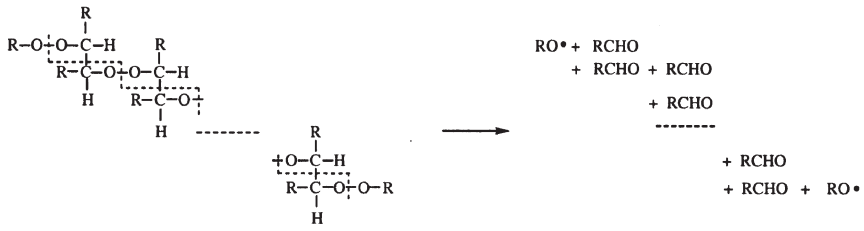
We propose a classification system for autocatalytic products. The first category is based on the mechanism of formation, which is divided into primary and secondary types. A primary-type mechanism means that the product is formed from species in the chain reaction without participation of the accumulated products, e.g., *via* a minor route of the chain reaction or the termination. A secondary-type mechanism means that the product is formed from an accumulated product. This type is further subdivided into two types: products formed with the participation of chain-reaction species, and those formed without such participation. The second category in our classification system is based on the stability of a substance, which can be deduced from its tendency to accumulate. A case in which almost all of the product is accumulated and only a small part participates in radical generation is found at the beginning of the scale on the stability of a substance; at the end is a product that decomposes to give radicals immediately after formation, resulting in almost no accumulation. There are many possible intermediate cases.

The substances obtained in this study are of the secondary type and have some tendency to accumulate. They may be responsible for a marked increase in rate at a late stage of autoxidation, as shown in Table 1 in the comparison between oxML2 (hydroperoxide level, 250 μmol/mL; autoxidation-accelerating activity, 7.44×10^{-5} Azo eq) and oxML3 (500 μmol/mL; 23.6×10^{-5} Azo eq); a remarkable increase in their accumulation was observed during this period.

A primary product with a tendency to accumulate is expected to be responsible for an increase in rate over all stages of autoxidation. If the working autocatalyst is of a type in which there is extensive accumulation, with only a small part participating in radical generation (i.e., close to the beginning of the scale in the second category of our classification system), the slope of the oxidation curve is expected to increase gradually from an initial value of 0; if it is at the other end of the scale (a tendency to immediate decomposition), the oxidation curve will be straight, as



SCHEME 1



SCHEME 2

the amount of the autocatalyst is not increased by accumulation, although it displays high activity. In our experiences involving the oxidation of the substrate pentyl linoleate in this study, carefully purified esters of linoleic acid resulted in straight-line oxidation courses, with positive slopes from the beginning.

What kind of compounds can exist as autocatalytic substances involving the latter type? Formation of the substances under discussion, a peroxide-linked dimer with two hydroperoxy groups, is thought to begin with the addition of a peroxy radical to a conjugated diene. In a similar mechanism, intramolecular addition of a linoleate peroxy radical to its conjugated diene can result in ring peroxidic compounds. In linolenate autoxidation, the intramolecular addition of peroxy radicals is known to occur (15,16). Another possibility is addition to minor conjugated polyene components of lipids such as conjugated polyene FA. These reactions may be important in the formation of autocatalytic substances; some of the substances formed may be unstable and thus appear near the end of our scale. The search for these substances is in progress.

Contrary to widely held opinion, the aldehydes hexanal, 2-heptenal, 2-octenal, and 2,4-decadienal were not formed from main-product hydroperoxide during linoleate autoxidation under mild conditions (7,17). The autocatalytic substances under discussion and those we expect to find share a structural feature that results in the joint formation of radicals and aldehydes during decomposition. Such decomposition may be an important route to aldehyde formation, and Miyashita *et al.* (18) reported that hydroxy aldehydic products were found in oxidation products of the dimer fraction described in Reference 11.

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Novel Imine from *Hemsleya macrocarpa* var. *clavata*

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ABSTRACT: The new substance hemsleyin imine A (**1**) was isolated along with with (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino)-octadecan-1,3,4-triol (**2**), (2*S*,3*R*,4*E*,8*E*)-1-*O*-β-D-glucopyranosyl-3-hydroxy-2-(2′*R*-hydroxypalmitoylamino)-4,8-octadecadiene (**3**) from the rhizomes of *Hemsleya macrocarpa* var. *clavata*. Their chemical structures were established mainly by spectral analysis and chemical evidence. Compound **1** possesses the skeleton of an imine moiety, which is novel in natural products.

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Hemsleya macrocarpa var. *clavata* (Cucurbitaceae), a perennial plant found in northwestern China, is used as folk medicine to treat bronchitis, bacillary dysentery, and tuberculosis (1). Cucurbitacin and triterpenoid glycosides have been isolated from some species of *Hemsleya* (2,3). During our investigations on *Hemsleya* plants (4–6), a novel imine of sphingolipids, hemsleyin imine A (**1**), was isolated along with two known compounds, (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino)-octadecan-1,3,4-triol (**2**) (7) and (2*S*,3*R*,4*E*,8*E*)-1-*O*-β-D-glucopyranosyl-3-hydroxy-2-(2′*R*-hydroxypalmitoylamino)-4,8-octadecadiene (**3**) (8) (see Fig. 1). Sphingolipids have been reported more often from higher fungi, such as the family Russulaceae (9,10), than from higher plants. In this work, compounds **1–3** were obtained for the first time from members of the Cucurbitaceae. This paper mainly describes the isolation and structural identification of the novel imine from the ethyl acetate fraction of the 90% ethanol extract of *H. macrocarpa* var. *clavata*.

EXPERIMENTAL PROCEDURES

Instrumentation. Melting points were obtained on an XRC-1 apparatus (Sichuan, People's Republic of China); IR spectra were obtained on a Bio-Rad FTS-135 spectrometer (Bio-Rad, Richmond, CA) with KBr disc; 1D- and 2D-NMR spectra were recorded on Bruker AV-400 and DBX-500 spectrometers (Karlsruhe, Germany), with trimethylsilane as internal standard. Chemical shifts (δ) are reported in ppm and coupling constants in hertz (Hz); mass spectra were determined on a VG-Auto Spec-3000 spectrometer.

Materials. Column chromatography (CC) was carried out on silica gel (200–300 mesh) and silica gel H (10–40 mesh). TLC was carried out on plates precoated with silica gel GF_{254} (Qingdao Haiyang Chemical Ltd., Qingdao, P.R. China).

Plant material. The aerial part of *H. macrocarpa* var. *clavata* was collected in Lincang County, Yunnan, China, in September 2001 and identified by Prof. Shukun Chen (Kunming Institute of Botany, Chinese Academy of the Sciences). A voucher specimen (K20010715) of this plant was deposited at the Kunming Institute of Botany, the Chinese Academy of Sciences.

Extraction and isolation. The dried and powdered plants (2.8 kg) were extracted with 90% ethanol at room temperature for 3 d. The filtrate was concentrated *in vacuo*, and the residue was partitioned between H_2O and ethyl acetate (AcOEt), then between H_2O and *n*-butanol. The AcOEt-soluble fraction (112 g) was subjected to CC (petroleum ether/acetone 50:1; 20:1; 10:1; 5:1, vol/vol) to afford several fractions. The fraction from petroleum ether/acetone 20:1 was further purified by CC (petroleum ether/AcOEt 10:1, vol/vol) to give compound **1** (30 mg). The fraction from petroleum ether/acetone 5:1 was separated by CC ($CHCl_3$/MeOH 8:1, vol/vol) to give the compound **2** (40 mg). The *n*-butanol fraction (90 g) was subjected to D101 column (Tianjin Chemical Factory, Tianjin, P.R. China) eluted with 20% EtOH, 40% EtOH, 60% EtOH, and 80% EtOH to give several fractions. The 80% EtOH fraction was purified by CC ($CHCl_3$/MeOH/H_2O 7:1:0.1, by vol) to afford compound **3** (23 mg).

*Hemsleyin imine A (**1**).* White powder, m.p. 64–65°C, $[\alpha]_D = -3.41$ ($c = 0.58$, $CHCl_3$). IR (KBr) ν_{max} cm^{-1}: 3315, 2923, 2857, 1738, 1656, 1636, 1533, 1465, 1380, 1077, 719. ^1H NMR ($CDCl_3$, 500 MHz), ^{13}C NMR ($CDCl_3$, 100 MHz) δ: see Table 1. EI-MS (70 eV) *m/z* (%): 607 $[M]^+$ (35), 579 $[M + H - CH_2OH + 2]^+$(100), 396 (25), 368 $[M - CH_3(CH_2)_{16}]^+$ (43), 340 $[M - CH_3(CH_2)_{16}CO]^+$, 313 (38), 267 $[CH_3(CH_2)_{16}CO]$ (45), 239 $[CH_3(CH_2)_{16}]^+$ (63), 98 (44), 57 (97). High resolution (HR)-EI-MS *m/z*: 607.5653 ($C_{39}H_{77}NO_3$, calc. 607.5963).

*(2S,3S,4R,2′R)-2-(2′-Hydroxytetracosanoylamino)-octadecan-1,3,4-triol (**2**).* White amorphous solid, m.p. 104–114°C, $[\alpha]_D = +9.4$ ($c = 2.5$, pyridine); IR (KBr) ν_{max} cm^{-1}: 3427–3245 (OH), 2920, 2850, 2486, 2395, 1619, 1552 (N–C=O), 1027, 720. EI-MS (70 eV) *m/z* (%): 683 $[M]^+$ (2), 665 $[M - H_2O]^+$ (20), 456 $[M - CH_3(CH_2)_{13}CHOH]^+$ (15), 439 [456 – OH]$^+$ (18), 384 $[CH_3(CH_2)_{21}CH(OH)CONH_2 + H]^+$ (25), 357

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Abbreviations: CC, column chromatography; DEPT, distortionless enhancement by polarization transfer; HMBC, heteronuclear multiple bond coherence; HR, high resolution.

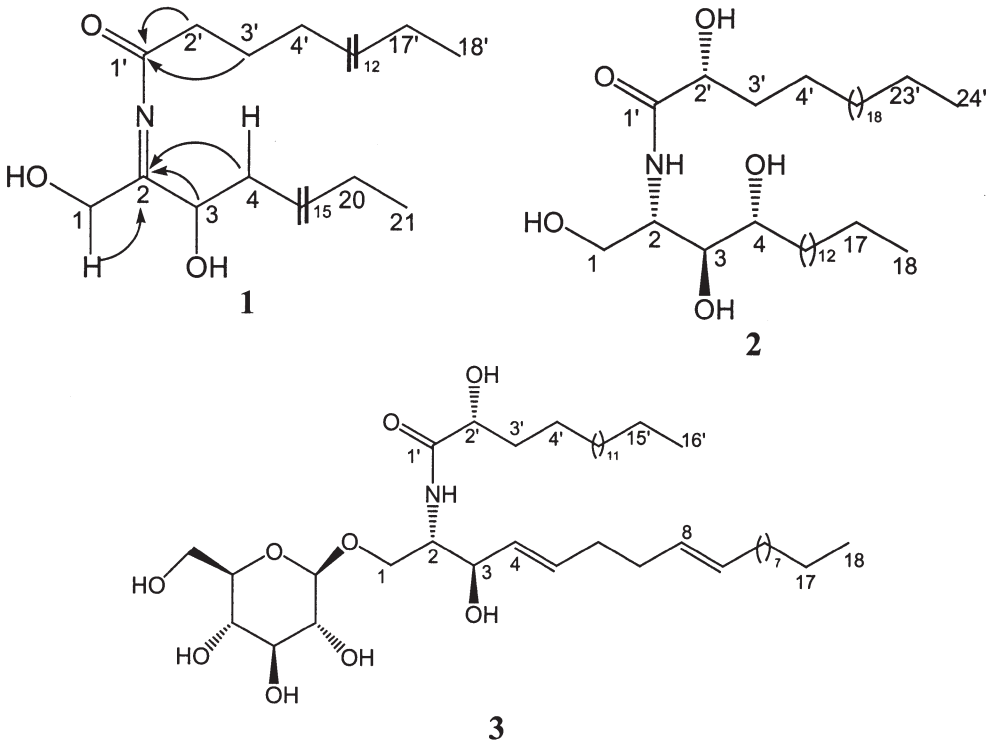

FIG. 1. Compounds isolated from *Hemsleya macrocarpa* var. *clavata*. (→) Heteronuclear multiple bond coherence.

$[M - CH_3(CH_2)_{21} - OH]^+$ (30), 320 (10), 227 (15); HR-EI-MS *m/z*: 683.6418 $[M]^+$ ($C_{42}H_{85}NO_5$, calc. 683.6428); the NMR and IR spectra of **2** were identical with those reported in the literature (7).

*(2S,3R,4E,8E)-1-O-β-D-Glucopyranosyl-3-hydroxy-2-(2′R-hydroxypalmitoylamino)-4,8-octadecadiene (**3**).* White amorphous solid, m.p. 130–131°C, $[\alpha]_D$ = +1.03 (*c* = 0.97, CH_3Cl). IR (KBr) ν_{max} cm^{-1}: 3450–3315 (OH; NH), 2920, 2850, 2486, 2395, 1738 (C=O), 1656 (C=C), 1636, 1533 (–NH–), 1027, 720. FABMS *m/z* (%): 712 $[M - H]^+$ (70), 688 $[M - Me]^+$ (35), 688 $[M - 192]^+$ (70), 255 $[M - 457]^+$ (13), 227 $[M - 485]^+$ (5), 153 $[M - 559]^+$ (100). HR-FABMS *m/z*: 712.5448 $[M - H]^-$ ($C_{40}H_{74}NO_9$, calc. 712.5443). The NMR and IR spectra of **3** were identical with those reported in the literature (8).

*Alkaline reaction of **1**.* To a solution of compound **1** (0.88 mg) dissolved in MeOH (2 mL) was added MeONa (1 mg). The mixture was allowed to stand for 2 d at room temperature. The reaction mixture was extracted with *n*-hexane to give the *n*-hexane extract. The EI-MS spectrum of the mixture offered characteristic MS ion peaks for the methyl ester of stearic acid at *m/z* (%): 298 (M^+, 0.5), 267 ($[M - OCH_3]^+$, 4), 253 (5), 239 (6), 225 (6), 211 (8), 197 (8), 183 (11), 169 (14), 155 (15), 141 (20), 127 (24), 113 (34), 99 (40), 85 (100), 71 (94), 57 (93).

RESULTS AND DISCUSSION

Compound **1** was obtained as a white powder. The molecular formula $C_{39}H_{77}NO_3$ was provided by the ion peak at *m/z* 607.5653 in the HR-EI-MS spectrum, combined with the ^{13}C distortionless enhancement by polarization transfer (DEPT)-NMR spectrum. The IR spectrum indicated the presence of the hydroxyl group (3315 cm^{-1}), carbonyl group (1738 cm^{-1}),

TABLE 1
NMR Data[a] of Hemsleyin Imine (1) in $CDCl_3$

Position	δ_C (DEPT)	δ_H (multi., *J* = Hz)	Position	δ_C (DEPT)	δ_H (multi., *J* = Hz)
1	62.1 (*t*)	4.22 (*dd*, 11.8, 4.0) 4.07 (*dd*, 11.8, 6.0)	21	14.1 (*q*)	0.80 (*t*, 6.8)
2	173.3 (*s*)	/[b]	1′	179.1 (*s*)	/
3	68.9 (*d*)	5.20 (*m*)	2′	34.1 (*t*)	2.25 (*m*)
4	33.9 (*t*)	2.25 (*m*)	3′	24.7 (*t*)	1.55 (*m*)
5	24.9 (*t*)	1.55 (*m*)	4′–17′	22.7–32.0 (*t*)	1.36–1.18 (*m*)
6–20	22.7–31.9 (*t*)	1.36–1.18 (*m*)	18′	14.1 (*q*)	0.80 (*t*, 6.8)

[a]DEPT, distortionless enhancement by polarization transfer; multi., multiplicity.
[b]No signal detected.

imine (3013, 1656 cm^{-1}), and aliphatic chains (2923, 2857, 719 cm^{-1}). The ^{13}C NMR spectrum (DEPT) showed the signals of two quaternary carbons at δ_C 179.1 and 173.3 (carbonyl and imine), only one oxymethine at δ_C 68.9, methylenes (including an oxymethylene), and methyl group δ_C 14.1, which suggested the presence of long aliphatic chains. The ^1H NMR data of **1** revealed the presence of two terminal methyls at δ_H 0.80 (6H, *t, J* = 6.8 Hz), methylene protons at δ_H 1.18–1.36, 1.54, 2.25 (64H, *brs*), one oxymethylene with two protons at δ_H 4.07 (1H, *dd*, *J* = 11.8, 6.0 Hz, H-1) and 4.22 (1H, *dd*, *J* = 11.8, 4.0 Hz, H-1), and one oxymethine proton vicinal to the imine bond at δ_H 5.20 (1H, *m*), which strongly indicated that **1** had an imide skeleton with long chains (7,9). In the ^{13}C NMR spectrum, C-1′ resonated at a relative low field (δ 175.6), suggesting that the imine bond was at C-2. The imine C-atom at C-2 was further confirmed by the heteronuclear multiple bond coherence (HMBC) correlations of H-1 (δ_H 4.07, *dd*, *J* = 11.8, 4.0 Hz, H-1); 4.22 (*dd*, *J* = 11.8, 6.0 Hz) with C-2 (δ_C 173.3, *s*) and C-3 (δ_C 68.9, *d*) and H-1, H-3 (δ_H 5.20, *m*) and H-4 (δ_H 2.25, *m*) with C-2. Furthermore, correlations between δ_C 173.3 (*s*, C-2) and H-1 (δ_H 4.22 and 4.07, 2H), H-3 (δ_H 5.20, *m,* 1H), H-4 (δ_H 2.25, *m*, 2H) were observed, which further confirmed that the imine C-atom was C-2. The correlation between H-3 (δ_H 5.20, *m,* 1H) and C-1 (δ_C 62.1, *t*) in HMBC suggested that the hydroxyl group was at C-3. Compound **1** could be considered to possess normal side chains because the carbon atom signals due to terminal methyl groups were observed at δ_C 14.1 (normal form) (11). Comparing optical rotation data (**1**, $[\alpha]_D$ = −3.41) with those of the literature (7,9) and considering the possible biosynthetic pathway, it is difficult for us to confirm the absolute configuration at C-3 of **1**. On the basis of the above-mentioned evidence, the structure of **1** was 2-octadecanoylimino-heneicosan-1,3-diol. Compound **1** is particularly interesting in view of the rare imine moiety in natural products.

Compounds **2** and **3** were identified by comparing their spectral and physical data with those reported (7,8).

ACKNOWLEDGMENTS

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Regulation of the α-Tocopherol Transfer Protein in Mice: Lack of Response to Dietary Vitamin E or Oxidative Stress

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ABSTRACT: The α-tocopherol transfer protein (TTP) plays an important role in the regulation of plasma α-tocopherol concentrations. We hypothesized that hepatic TTP levels would be modulated by dietary vitamin E supplementation and/or by oxidative stress. Mice were fed either a High E (1150 mg *RRR*-α-tocopheryl acetate/kg diet) or a Low E (11.5 mg/kg diet) diet for 2 wk. High E increased plasma and liver α-tocopherol concentrations approximately 8- and 40-fold, respectively, compared with Low E-fed mice, whereas hepatic TTP increased approximately 20%. Hepatic TTP concentrations were unaffected by fasting (24 h) in mice fed either diet. To induce oxidative stress, chow-fed mice were exposed for 3 d to environmental tobacco smoke (ETS) for 6 h/d (total suspended particulate, 57.4 ± 1.8 mg/m3). ETS exposure, while resulting in pulmonary and systemic oxidative stress, had no effect on hepatic α-tocopherol concentrations or hepatic TTP. Overall, changes in hepatic TTP concentrations were minimal in response to dietary vitamin E levels or ETS-related oxidative stress. Thus, hepatic TTP concentrations may be at sufficient levels such that they are unaffected by either modulations of dietary vitamin E or by the conditions of environmentally related oxidative stress used in the present studies.

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The α-tocopherol transfer protein (TTP) is a 33-kDa protein that is predominantly expressed in the mammalian liver (1) but which has also been detected in the rat brain, spleen, lung, and kidney (2); in the human brain (3); and in mouse liver, brain (4,5); and uteri (6). Based on sequence analysis, TTP is classified as a member of the SEC14-like protein family, with a characteristic CRAL_TRIO lipid-binding domain (7,8). In the closed TTP crystal structure, a mobile helical surface segment seals the hydrophobic binding pocket (9,10) and is responsible for the TTP selectivity for α-tocopherol (11).

Mutations in the TTP gene in humans [ataxia with vitamin E deficiency; (12)] are associated with extraordinarily low plasma E concentrations (13). In addition, in TTP-knockout and heterozygote mice, plasma α-tocopherol concentrations in plasma are 5 and 50% those of the wild-type mice, respectively (14,15). Clearly, TTP plays an important role in the regulation of plasma α-tocopherol concentrations. However, studies conducted on the regulation of TTP in response to dietary vitamin E intake have yielded conflicting results.

Kim *et al.* (16) showed that levels of rat hepatic TTP mRNA and protein were lower in response to high levels of dietary vitamin E (600 mg DL-α-tocopherol/kg diet) compared with rats fed the control diet (50 mg DL-α-tocopherol/kg diet). In rats that were fed vitamin E-deficient diets, TTP mRNA was higher, but protein levels were unchanged compared with controls. In contrast, Shaw and Huang (17) showed that in vitamin E-deficient rats, hepatic TTP was lower compared with rats fed control diets (50 mg all-*rac*-α-tocopheryl acetate/kg diet) but control and vitamin E-supplemented (5,000 mg all-*rac*-α-tocopheryl acetate/kg diet) rats had similar TTP concentrations. No changes in TTP mRNA were observed. Last, Fechner *et al.* (18) showed that rat hepatic TTP mRNA levels were unchanged by dietary vitamin E depletion and fasting (24 h). However, refeeding α-tocopherol to vitamin E-depleted rats followed by an additional 24-h fast increased the expression of TTP mRNA about sevenfold; however, TTP protein levels were not measured. Since α-tocopherol is secreted from the liver and associated with plasma VLDL (19), fasting, which is known to be associated with low levels of VLDL, might be expected to be accompanied by reduced liver cell release of α-tocopherol to plasma and modulations of TTP levels. Overall, no consistent changes in TTP protein in response to different dietary levels have been reported. However, oxidative stress has been suggested to increase hepatic TPP (20).

Environmental tobacco smoke (ETS) contains highly reactive components, many of which cause oxidative stress and activate host inflammatory-immune processes (21,22). Exposure of plasma to gas-phase cigarette smoke *in vitro* causes depletion of antioxidants such as vitamins C and E (23). Since smokers have a higher rate of vitamin E disappearance (24,25) despite maintaining normal or somewhat depleted plasma α-tocopherol concentrations (26–30), we hypothesized that the oxidative stress of cigarette smoke exposure might increase hepatic TTP concentrations. The purpose of the present study was to evaluate whether hepatic TTP in mice is regulated in response to variations in dietary vitamin E, fasting, or ETS exposure.

The first and second investigators contributed equally to this work.

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Abbreviations: α-CEHC, α-carboxyethyl hydroxychroman; DNPH, dinitrophenylhydrazine; ETS, environmental tobacco smoke; 4-HNE, 4-hydroxy-2-nonenal; TSP, total suspended particulates; TTP, α-tocopherol transfer protein.

EXPERIMENTAL PROCEDURES

TTP regulation by dietary vitamin E: Animals and dietary treatment. The protocols for the care and use of animals were approved by the Institutional Care and Use Committee at Oregon State University or at the University of California at Davis. For the dietary experiments, male C57BL/6 mice that weighed ~20–22 g (~7 wk of age) were purchased from Simonsen Laboratories, Inc. (Gilroy, CA). Mice were housed individually in stainless-steel mesh cages in a room maintained at 22°C and 40–60% humidity. Animals were weighed and then randomly assigned to the High E ($n = 6$) or Low E ($n = 7$) diet, respectively (diet study 1). Mice had free access to diet and water throughout the experiments. Animal weight and diet consumption was recorded twice per week. On day 15, mice were anesthetized by CO_2 inhalation. Blood was drawn from the heart into a 2-mL purple-topped vacutainer tube (1 mg/mL EDTA), mixed, separated by centrifugation, and the plasma was stored at −80°C. The liver was rinsed with ice-cold saline, blotted, weighed, immediately frozen in liquid nitrogen, and stored at −80°C. α-Tocopherol was extracted from the plasma and liver and analyzed as described by Podda *et al.* (31). The same protocol was followed for a second group of animals (High E: $n = 6$, and Low E: $n = 6$) except that half ($n = 3$) of the animals in each diet group were fasted for 24 h prior to sacrifice (diet study 2).

The diet composition for the two diet studies is given in Table 1. Diets were based on AIN 93M (32) and were prepared to contain *RRR*-α-tocopheryl acetate (ADM Nutraceuticals, Decatur, IL), either 11.5 mg *RRR*-α-tocopheryl acetate/kg diet (Low E) or 1150 mg *RRR*-α-tocopheryl acetate/kg diet (High E). The diet composition was analyzed in our laboratory to confirm the dietary vitamin E contents. The Low E diet contained half of the recommended vitamin E for mice [22 mg α-tocopheryl acetate per kg diet (33). The Low E diet provided the animals with approximately 2.3 mg of *RRR*-α-tocopheryl acetate/d. This diet was designed to provide the equivalent of approximately 100 mg *RRR*-α-tocopherol per day for a 70-kg human (based on an average mouse intake of 4 g diet per day and an average weight of 20 g).

TABLE 1
Composition of Low and High E Diets[a]

Ingredients	Experimental diets (g per kg diet)	
	Low E	High E
Vitamin-free casein	140	140
L-Cystine	1.8	1.8
Cornstarch	455.9	454.7
Maltodextrin	155	155
Sucrose	109.8	109.8
Cellulose	50	50
Tocopherol-stripped corn oil	40	40
RRR-α-tocopheryl acetate in tocopherol-stripped corn oil	0.012	1.2
Mineral mix (AIN-93M-MX)	35	35
Vitamin mix[b] (AIN-93M-VX)	10	10
Choline bitartrate	2.5	2.5

[a]Diets were prepared by Harlan Teklad (Madison, WI).
[b]Vitamin E was excluded from the vitamin mix and replaced with sucrose.

TABLE 2
Composition of Diet[a] Fed to Environmental Tobacco Exposed (ETS) Mice

Ingredients	g per kg diet
Protein	180
L-Cystine	25
Fat (ether extract)	60
Fibre (crude)	50
Mineral (Ash)	80
Vitamin E (IU/kg)	45

[a]Commercially available through Purina Lab Diet, diet #5K52 (www.labdiet.com)

TTP regulation by oxidative stress. (i) Animals and experimental treatment. C57BL6 mice (~12 wk of age) were exposed to ETS (ETS: $n = 8$; filtered air: $n = 8$, n indicated in the figure legends; insufficient sample was available for use in all assays) for 3 consecutive days. Mice exposed to ETS were purchased from Jackson Laboratory (Bar Harbor, ME). They were fed the vendor's diet (Purina 5K52; LabDiet, Richmond, IN) containing 45 IU/kg α-tocopherol (Table 2). At the end of the experimental treatment (day 3), animals were anesthetized with an intraperitoneal injection of 120 mg sodium pentobarbital/kg body weight. Sample collection, storage, and analysis were conducted as described for the diet studies.

(ii) Exposure of mice to cigarette smoke. Exposures of mice to ETS were conducted at the Institute of Toxicology and Environmental Health, University of California at Davis. The exposure system and methods used to generate ETS have been described elsewhere (34,35). Briefly, mice were exposed to a mixture of mainstream and sidestream cigarette smoke (aged and diluted) from 1R4F reference research cigarettes (1.2 mg nicotine/cigarette) obtained from the Tobacco and Health Research Institute of the University of Kentucky. An automatic cigarette-smoking machine generated cigarette smoke in a staggered manner at the rate of a 35-mL puff over a 2-s duration each min. During this period of "passive" smoking, mice were exposed to total suspended particulates (TSP, 57.4 ± 1.8 mg/m^3), carbon monoxide (262 ± 6 ppm), and nicotine (10.5 ± 1.0 mg/m^3) for 6 h/d × 3 d.

Western blot analysis for TTP and 4-hydroxy-2-nonenal (4-HNE). To obtain the soluble liver fraction for Western blot analysis, a portion of liver (frozen in liquid nitrogen on the day of sacrifice) from each animal was homogenized in PBS (0.1 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4) (20%, wt/vol). The homogenate was centrifuged at 20,000 × g for 30 min at 4°C and the resulting low-speed supernatant was centrifuged at 100,000 × g for 60 min at 4°C to obtain the high-speed supernatant. The high-speed supernatant was stored at −80°C until Western blot analyses were performed. The protein concentration in the liver supernatant samples was determined by a commercially available kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA).

Rabbit antirat TTP serum was a gift from Dr. Robert Farese (Gladstone Institute of Cardiovascular Disease and the

Cardiovascular Research Institute, Department of Medicine, University of California at San Francisco, San Francisco, CA). The polyclonal antibody was raised against a peptide of the C-terminus 1–21 amino acids of rat TTP and cross-reacted with mouse liver TTP (14). Proteins (40 mg per lane for ETS or 100 mg per lane for diet studies) were then subjected to electrophoretic separation under denaturing and reducing conditions and transferred onto a nitrocellulose membrane. Immunoreactive protein was detected by chemiluminescence by using goat antirabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) with exposure to Kodak X-OMAT XRP film (Rochester, NY). Quantitative densitometric analysis of the image was performed using an AlphaImager™ 2000 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA). The O.D. value represents the sum of all pixels minus the background within the specified area. For all experiments, a single band was detected for TTP corresponding to 32 kDa. This M.W. is consistent with the published apparent M.W. for mouse TTP (14).

Lipid oxidation and environmental pollutants result in the production of unsaturated aldehydes such as 4-HNE. Lung and liver homogenates from ETS and air-exposed animals were separated by electrophoresis (10% SDS-gel, reducing conditions) and after transfer onto polyvinylidene difluoride membrane probed with rabbit anti4-HNE antibody (Oxis International Inc., Portland, OR, 1:1000). Immunoreactive protein was visualized as described above.

Protein carbonyls in bronchoalveolar lavage fluid. Oxidative modification of proteins in mouse lung lavage was determined using an ELISA method described previously (36). Briefly, after derivatization of carbonyl groups with dinitrophenylhydrazine (DNPH), proteins were adsorbed onto 96-well ELISA plates, captured with a commercially available anti-DNPH antibody, and detected with a horseradish peroxidase /hydrogen peroxide, phenylenediamine system (37).

Statistical analysis. Data are expressed as mean ± SD and were analyzed for statistical significance by Student's *t*-test, or ANOVA and Fisher's LSD using StatView 5.0 (SAS Institute, Inc., Cary, NC). Liver α-tocopherol concentrations were log transformed to normalize the variances between groups for the two diet studies. Differences were considered significant at $P < 0.05$.

RESULTS

Growth and food intake. In the diet studies, final body weights, weight gains (g/d), and diet consumed (g/d) were similar for all animals, except for the weight gain in diet study 1 (Table 3). Mice fed the High E diet gained more weight than those fed the Low E diet ($P = 0.02$) because of an initial low weight gain in one animal in the Low E group. Weight gain for this animal was similar to that of the other mice at the time of sample collection. In mice fasted for 24 h, livers weighed more in nonfasted than in fasted mice, irrespective of whether the mice were fed the High E ($P = 0.0013$) or Low E diet ($P = 0.0004$).

Plasma and hepatic vitamin E and TTP levels. (i) Responses to dietary vitamin E. Mice fed the High E diet had hepatic α-tocopherol concentrations ~40 times higher ($P < 0.0001$) and plasma α-tocopherol concentrations ~8 times higher ($P < 0.0001$) than those of mice fed the Low E diet (Figs. 1, 2). In the animals fed the Low E diet that were fasted for 24 h prior to sacrifice, no effect of fasting was observed on either plasma or hepatic α-tocopherol concentrations (Fig. 2). However, in mice fed the High E diet, fasting significantly decreased hepatic ($P = 0.003$) and plasma α-tocopherol concentrations ($P = 0.0003$) (Fig. 2).

In contrast to the 40-fold increase in hepatic α-tocopherol, mice fed the High E diets had hepatic TTP levels that were only 20 ($P = 0.02$) and 25% ($P = 0.002$) higher compared with those fed the Low E diet in studies 1 and 2, respectively (Figs. 1, 2). Hepatic TTP levels were unaffected by fasting in mice fed either the High E or Low E diet (Fig. 2).

(ii) Responses to oxidative stress. Exposure of mice to ETS for 3 d caused increased oxidative stress to proteins and lipids. Figure 3A shows increased formation of protein carbonyls in the lung lavage of animals exposed to ETS ($P = 0.007$). These results and the greater amount of 4-HNE adducts in lung tissue with ETS exposure vs. air ($P < 0.01$) (Fig. 3B) demonstrate pulmonary oxidative stress. The increase in lung 4-HNE in ETS-exposed mice seems to be transient, as adduct formation returned to control levels on day 3. Exposure to ETS also caused a significant increase in 4-HNE in liver ($P = 0.005$), indicative of systemic oxidative stress. Furthermore, exposure to ETS was associated with a 40% decrease in the lung α-tocopherol concentration ($P = 0.001$; Fig. 4A). However, hepatic α-tocopherol concentrations (Fig. 4B) and TTP levels were unaffected by ETS exposure (Fig. 4C).

TABLE 3
Growth and Dietary Intake Parameters of Mice Fed Low and High E Diets[a]

	Experiment 1		Experiment 2			
			Low E		High E	
Diet	Low E	High E	Fasted	Nonfasted	Fasted	Nonfasted
Final weight (g)	23.1 ± 1.1[#]	24.8 ± 1.2*	24.4 ± 1.3	23.4 ± 1.3	25.5 ± 0.8	25.9 ± 2.2
Liver weight (g)	1.5 ± 0.1	1.6 ± 0.2	0.9 ± 0.2[§]	1.4 ± 0.0[†]	0.9 ± 0.1[§]	1.5 ± 0.1[†]
Gain/d (g)	0.2 ± 0.03	0.2 ± 0.03	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.05	0.2 ± 0.10
Diet/d (g)	3.7 ± 0.3	3.9 ± 0.2	3.8 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	3.7 ± 0.2

[a]Values are means ± SD for High E ($n = 6$) and Low E ($n = 7$). For experiment 1, the final weight was significantly greater in mice fed the High E (*) vs. the Low E (#) diet ($P = 0.02$). For experiment 2 ($n = 3$ for each treatment), in mice fasted for 24 h, liver weights were significantly higher for nonfasted (†) compared with fasted mice [High E: $P = 0.0013$ (§); Low E: $P = 0.0004$ (§)] by ANOVA and Fisher's LSD test.

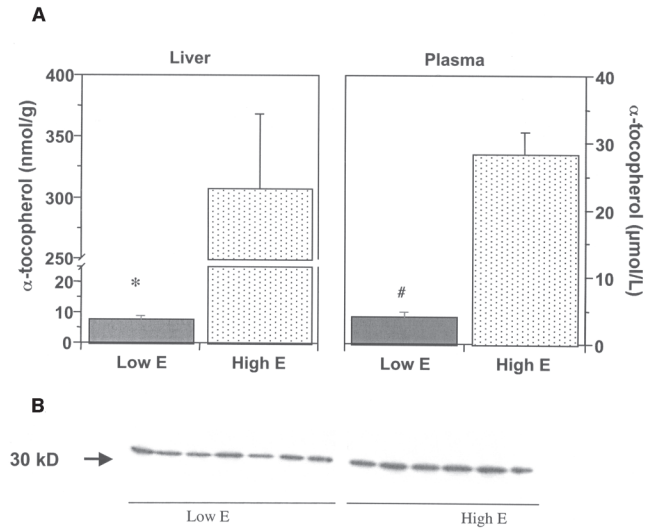

FIG. 1. Plasma and hepatic vitamin E levels and α-tocopherol transfer protein (TTP) concentrations in response to dietary vitamin E (A) Mice fed High E ($n = 6$) compared with Low E ($n = 7$) diets showed higher hepatic (*) and plasma (#) α-tocopherol concentrations, respectively (means ± SD, $P \leq 0.0001$). (B) Western blots of hepatic TTP from the livers of mice described in (A). Hepatic TTP levels were 20% higher ($P = 0.02$) in mice fed the High E diets (30,864 ± 5,199 pixels/mm^2) compared with those fed the Low E diet (24,570 ± 3,239 pixels/mm^2).

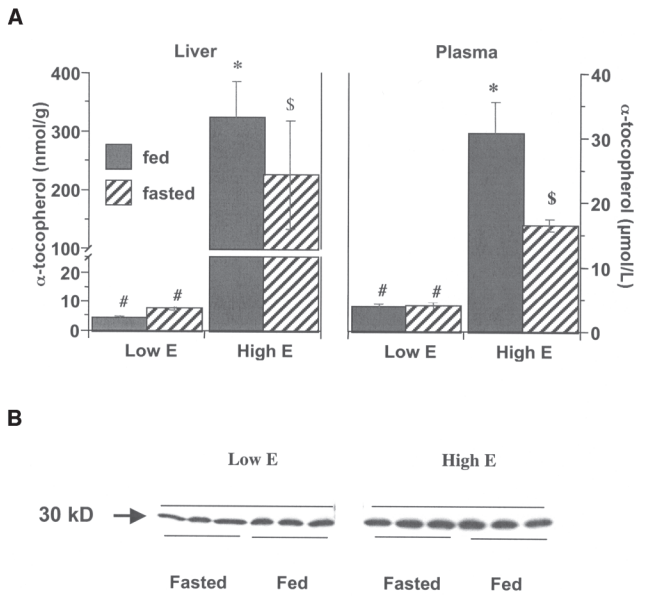

FIG. 2. Effect of fasting on plasma and hepatic vitamin E levels and TTP protein concentrations. (A) Hepatic and plasma α-tocopherol concentrations in mice (means ± SD, $n = 3$ for each of the four treatments) fed Low or High E diets followed by a 24-h fast or nonfast. Hepatic and plasma α-tocopherol concentrations were significantly lower in the Low E (#) compared with the High E diet-fed animals ($P < 0.0001$, diet effect) (*,$). Mice fed the High E diet that were fasted had significantly lower hepatic and plasma α-tocopherol concentrations than nonfasted mice [$P = 0.003$ for hepatic (* vs. $), and $P = 0.0003$ for plasma α-tocopherol (* vs. $), ANOVA and Fisher's LSD test]. (B) Western blots of hepatic TTP from the livers of mice described in (A). Hepatic TTP levels were 25% higher ($P = 0.002$) in mice fed the High E diets (40,404 ± 3,920) compared with those fed the Low E diet (29,545 ± 4,431). Hepatic TTP levels were unaffected by fasting. For abbreviation see Figure 1.

DISCUSSION

Hepatic TTP levels in mice were approximately 20% higher in response to the 100-fold greater dietary α-tocopherol levels in the High E compared with the Low E diet. This relatively small increase in TTP occurred despite a 40-fold increase in hepatic α-tocopherol in mice fed the High E diet. Plasma α-tocopherol levels increased only eightfold in the nonfasted mice fed the High E compared with the Low E diet (Figs. 1, 2). Clearly, plasma α-tocopherol concentrations do not increase to the extent they increase in the liver in response to dietary vitamin E. These data suggest that although liver α-tocopherol increased, plasma α-tocopherol levels were limited by the marginal increase in hepatic TTP and/or by the capabilities of liver-secreted lipoproteins to accept additional amounts of α-tocopherol (38).

Hepatic TTP levels were 20 and 25% higher in diet studies 1 and 2, respectively, in mice fed the High E diet compared with those fed the Low E diet. Although the levels of dietary vitamin E in our study were not identical to those used by Shaw and Huang (17), the direction and magnitude of changes in hepatic TTP levels were similar between the two studies. The authors (17) observed that TTP levels were *ca.* 25% higher in mice fed vitamin E-containing diets (for both their control and High E diets) compared with those fed a vitamin E-deficient diet, whereas no changes were observed in TTP mRNA levels (17). Using gene chip microarrays, Barella *et al.* (39) have also found a lack of significant difference in hepatic TTP mRNA expression in rats fed vitamin E-sufficient vs. vitamin E-deficient diets. Although Kim *et al.* (16) used diets and an animal species (rat) similar to those used by Shaw and Huang (17), the responses to vitamin E deficiency and supplementation did not agree for either hepatic TTP or TTP mRNA levels between the two studies. Reasons for these discrepancies have not been determined and warrant further investigation.

Fechner *et al.* (18) saw a significant increase in TTP mRNA levels in rats that consumed a vitamin E-deficient diet for 5 wk and were then fasted, refed vitamin E, and fasted again. However, this effect of fasting was evaluated only in animals fed a vitamin E-deficient diet and not in animals receiving dietary vitamin E. In contrast, we found that fasting for 24 h prior to sacrifice had no effect on the regulation of hepatic TTP levels in mice fed either low or high levels of dietary vitamin E. The significant changes in TTP mRNA levels observed by Fechner *et al.* (18) may be due to changes in vitamin E status or to changes in the animals' feeding vs. fasting state. Hormonal signals or aspects of the diet other than vitamin E levels may also regulate hepatic TTP levels (17).

Although no changes in hepatic TTP levels occurred with fasting, we observed lower hepatic and plasma α-tocopherol concentrations in fasted compared with nonfasted animals fed the High E diet, but not the Low E diet. Fasted animals that are receiving a High E diet will have a dramatic decrease in food

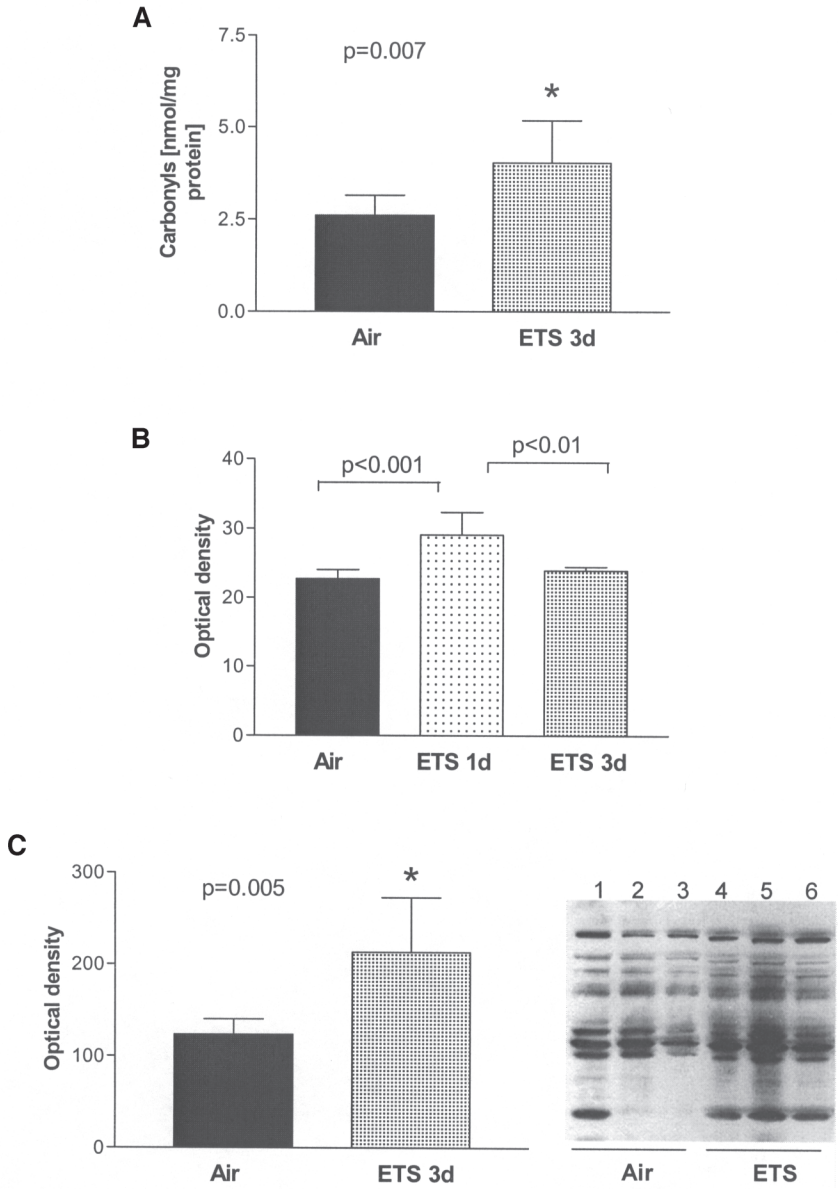

FIG. 3. Pulmonary and systemic oxidative stress in environmental tobacco smoke (ETS)-exposed mice. (A) Protein carbonyls in the lung lavage of mice exposed to ETS (3 d) vs. air (mean ± SD, $n = 8$). The protein carbonyl concentration was significantly higher in ETS-exposed animals ($P = 0.007$, Student's *t*-test). (B) 4-Hydroxy-2-nonenal (4-HNE) adduct formation in lung tissue of mice exposed to ETS (1 d and 3 d) vs. air (mean ± SD, $n = 8$). 4-HNE formation significantly increased after one day of ETS exposure but returned to control values by day 3. (O.D. 22.8 ± 1.2, 29.1 ± 3.2, and 23.9 ± 0.5 for air, ETS 1 d, and ETS 3 d, respectively, $P < 0.01$, ANOVA). (C) 4-HNE adduct formation in the liver tissue of mice exposed to ETS (3 d) vs. air (mean ± SD, $n = 6$). 4-HNE formation significantly increased after 3 d of ETS exposure (O.D. 123.6 ± 16.4 vs. 213.2 ± 59.4, $P = 0.005$, Student's *t*-test). A representative blot of liver homogenates from animals exposed to ETS and filtered air is shown (air: lanes 1–3; ETS: lanes 4–6).

intake and decreased delivery of vitamin E to both the liver and plasma. Increased metabolism of α-tocopherol may also play a role in the decreased hepatic and plasma α-tocopherol concentrations in these animals. Indeed, serum concentrations of α-carboxyethyl hydroxychroman (α-CEHC), a metabolite of α-tocopherol, have been shown to increase ~19-fold in individuals supplemented with 500 IU *RRR*-α-tocopherol per day for 29 d compared with unsupplemented individuals (40). Urinary α-CEHC is detectable at "low" vitamin E intakes (~5–10 mg/d) (41,42) in unsupplemented individuals and increases ~20-fold with supplementation of 800 mg/d (43). Hence, hepatic metabolism of α-tocopherol to α-CEHC in the mice may have been up-regulated with the high dietary levels of vitamin E.

We also considered that increased oxidative stress might up-regulate TTP. ETS exposure causes oxidative stress both because of the radicals in the smoke and through induction of airway- and systemic inflammatory-immune processes (22). Using deuterium-labeled vitamin E, Traber *et al.* (24) and

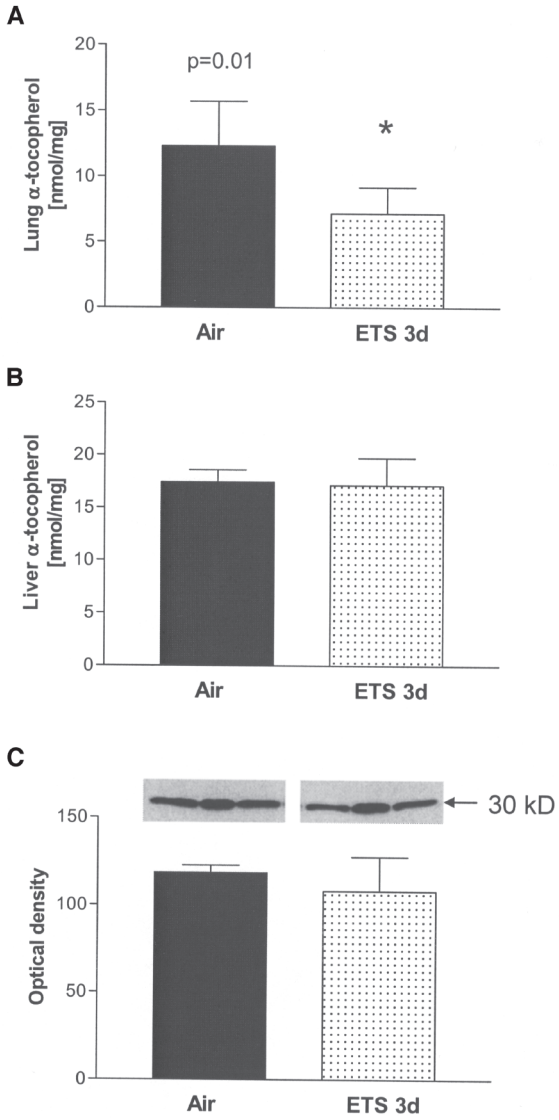

FIG. 4. Effect of ETS on pulmonary and hepatic vitamin E levels and TTP protein concentrations. (A) Pulmonary α-tocopherol concentrations in mice exposed to ETS (3 d) vs. air (mean ± SD, $n = 6$). Lung α-tocopherol concentrations decreased after 3 d of ETS exposure ($P = 0.001$, Student's *t*-test). (B) Hepatic α-tocopherol concentrations in mice exposed to ETS vs. air (mean ± SD). For hepatic tissue, ETS: $n = 5$, and air: $n = 4$. Hepatic α-tocopherol concentrations were similar by Student's *t*-test. (C) Western blots of hepatic TTP in mice exposed to ETS vs. air. No significant changes in TTP protein expression were observed ($P = 0.4$). Blots are of three representative liver homogenates from animals exposed to either ETS or air for 3 d. For abbreviations see Figures 1 and 3.

Bruno *et al*. (25) showed that cigarette smokers had a higher rate of vitamin E disappearance, yet total plasma α-tocopherol concentrations were similar in smokers and nonsmokers. We therefore hypothesized that the oxidative stress of cigarette smoking might increase hepatic TTP. Higher TTP levels could then potentially maintain plasma α-tocopherol. ETS-exposed mice exhibited increases in the oxidative modification of proteins (carbonyl formation) and lipids (HNE adducts) and a decrease in lung α-tocopherol. However, ETS exposure did not change hepatic α-tocopherol or up-regulate hepatic TTP (Fig. 4). Our exposure protocol mimics an acute ETS exposure. Although we showed increased formation of HNE adducts in the liver, it is possible that the ETS exposure regime [3 d (6 h/d) at 60 mg^3 TSP] used in this study did not cause a sufficient amount of systemic oxidative stress to deplete α-tocopherol stores and increase hepatic TPP expression. We did show, however, that exposure to ETS caused increased oxidative stress in the lung.

Thus, TTP plays an important role in the regulation of plasma levels of vitamin E. Together with Shaw and Huang (17) and Barella *et al.* (39), our study shows that the changes in hepatic TTP levels are minimal compared with changes in dietary or hepatic vitamin E levels. TTP appears to be normally expressed at sufficient levels to aid trafficking of newly absorbed dietary vitamin E. In our study, mice consuming the low vitamin E diet received half of the recommended vitamin E for mice [11.5 instead of 22 mg *RRR*-α-tocopheryl acetate/kg chow; (33)], similar to the low vitamin E diets used by others (44). TTP expression may therefore be optimized for sufficient vitamin E transport even under dietary vitamin E restrictions.

Under the conditions of oxidative stress used in this study, TTP was not up-regulated to compensate for the higher turnover or oxidative loss of vitamin E. Again, this may be due to optimal levels of TTP in the liver. However, we cannot exclude the possibility that higher levels of oxidative stress result in significant modulations in liver TTP levels. Therefore, additional studies at lower dietary and hepatic α-tocopherol concentrations are warranted.

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Effects of Orlistat Therapy on Plasma Concentrations of Oxygenated and Hydrocarbon Carotenoids

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ABSTRACT: Orlistat is a lipase inhibitor that is applied for treating obesity. Lipases are required for digestion and absorption of dietary lipids and fat-soluble vitamins and carotenoids. The aim of this study was to compare the effects of orlistat therapy on plasma concentrations of oxygenated (β-cryptoxanthin, lutein/zeaxanthin) and hydrocarbon (α-, β-carotene, lycopene) carotenoids. Six patients with a body mass index (BMI) ≥ 30 kg/m^2 received 360 mg/d orlistat over 4.5 mon. Plasma carotenoid concentrations were determined at baseline (T_0) and after 3 (T_3) and 4.5 mon ($T_{4.5}$) along with anthropometric, dietary, and biochemical indices, including plasma lipids, retinol, α- and γ-tocopherols, and FA. Baseline BMI was 32.7 ± 1.97 kg/m^2. Five of six patients lost weight; the average weight loss was 3.6 ± 2.4% ($P = 0.47$). There were no significant changes in dietary carotenoid intakes. In contrast, plasma α- and β-carotene concentrations decreased significantly from T_0 to $T_{4.5}$ by 45% ($P = 0.006$) and 32% ($P = 0.013$), respectively. Plasma lycopene decreased from T_0 to T_3 but increased again from T_3 to $T_{4.5}$, while β-cryptoxanthin and lutein/zeaxanthin concentrations did not change. There were no significant alterations in tocopherol, retinol, and FA concentrations. In conclusion, even though weight loss was not significant, orlistat therapy was associated with significant decreases in plasma concentrations of the highly lipophilic hydrocarbon carotenoids, α- and β-carotene.

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The prevalence of overweight and obesity has grown progressively over the past two decades in both developing and industrial countries (1). Since 1991, the proportion of obese inhabitants increased from 12 to 21% in the United States, and there are 300 million obese adults worldwide (1,2). Given the strong association between obesity and several chronic diseases such as cardiovascular disease and diabetes mellitus (3), these changes will have major health implications in the near future.

One of the options for long-term treatment of obesity is orlistat, a potent inhibitor of gastric and pancreatic lipases. Lipases hydrolyze TG into absorbable MG and FFA and are thus instrumental in the digestion and absorption of dietary fats from the gastrointestinal tract (4). Orlistat binds to the active site of lipases, resulting in the inhibition of enzyme activity and, subsequently, impaired formation of mixed micelles. Along with impaired absorption of dietary fat, the absorption of fat-soluble vitamins (5), carotenoids (6), and dietary FA (7) is affected.

The absorption of highly lipophilic compounds such as carotenoids is impaired in patients with clinically overt fat malabsorption, e.g., in patients with cystic fibrosis (8), and in the presence of minimally impaired lipase activity (9). Depending on the chemical structure and subsequent lipophilicity of different carotenoids, the absorption may be affected to different extents. As a consequence, the oxygenated carotenoids β-cryptoxanthin, lutein, and zeaxanthin may be more easily absorbed than the hydrocarbon carotenoids α- and β-carotene and lycopene (10,11).

Different carotenoids exert different biological actions and functions, including antioxidant and immunoenhancing effects, protection against cardiovascular disease and certain types of cancer (lycopene for prostate cancer), and maintenance of normal vision (lutein for age-related macular degeneration) (12). However, the relative importance of the individual carotenoids has not been clearly established.

The aim of our study was to investigate the effects of treatment with the lipase inhibitor orlistat on carotenoid status in general and, more specifically, to compare the effects on hydrocarbon carotenoids with those on oxygenated carotenoids. In addition, the effects on other fat-soluble compounds such as retinol, α- and γ-tocopherol, and FA were analyzed.

EXPERIMENTAL PROCEDURES

Patients. Consecutive patients who attended the Outpatient Clinic for Diabetes and Metabolism at the Department of Internal Medicine, Medical University of Graz, and who had a body mass index (BMI) > 30 kg/m^2 were eligible for enrollment in the study. Patients were excluded if one or more of the following criteria were present: pregnancy, lactation, cholestasis, chronic malabsorption, and chronic alcoholism. The study protocol was approved by the Ethics Committee of the Medical University of Graz, and written informed consent was obtained from the patients. The six patients enrolled included five postmenopausal women and one man, aged 59.5 ± 3.78 yr (min, 54 yr; max, 65 yr). One patient smoked on average four cigarettes per day, while the others were nonsmokers. Two patients suffered from combined hyperlipidemia and showed elevated TG and LDL cholesterol concentrations. Demographic data are presented in Table 1.

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Abbreviations: BMI, body mass index; FFQ, food frequency questionnaire.

TABLE 1
Anthropometric Data and Serum Cholesterol Concentrations (mean ± SD) During Orlistat Therapy[a] (*n* = 6)

	Time points		
	T_0	T_3	$T_{4.5}$
Weight (kg)	95.2 ± 11.0	91.4 ± 10.4	91.8 ± 10.7
BMI (kg/m^2)	32.7 ± 1.97	31.4 ± 1.48	31.5 ± 1.65
Waist (cm)	110 ± 4.51	106 ± 7.82	107 ± 4.90
Hip (cm)	121 ± 5.32	119 ± 4.27	119 ± 4.72
Waist/hip ratio	0.912 ± 0.068	0.887 ± 0.082	0.898 ± 0.066
Body fat (%)	41.9 ± 4.62	42.1 ± 3.78[b]	43.2 ± 2.74[b]
Total cholesterol	7.77 ± 1.18	7.15 ± 0.969	7.23 ± 0.492

[a]There were no significant differences between T_0, T_3, and $T_{4.5}$ for any of the variables studied. BMI, body mass index.
[b]n = 5.

Study design. In a single-center observational study, patients received 120 mg orlistat three times per day for 4.5 mon in addition to dietary advice and recommendations regarding physical activity as part of an established intervention program. Patients were advised to take the medication along with each fat-containing main meal during or up to 1 h after the meal. Patients were not allowed to take any vitamin and carotenoid supplements 2 mon prior to and during the trial. They were recommended to follow a weight-reducing diet including ≤30% of calories from fat, 10–15% from protein, and ≥50% from carbohydrates. Patients were monitored at the monthly visits for their dietary intake. Compliance with the study medication was checked by interview and pill count. The study period was based on the usual length of coverage of costs of obesity treatment with orlistat for patients with a BMI > 30 kg/m^2 by the major local health care provider.

Anthropometric assessment. At T_0, T_3, and $T_{4.5}$ anthropometric variables including weight, height, waist and hip circumference were measured, and the waist/hip ratio was calculated. Body fat was determined by bioimpedance analysis (BIA) using Bodystat Quadscan 4000 (Eumedics, Purkersdorf, Austria).

Dietary assessment. At T_0 patients completed a food frequency questionnaire (FFQ) for assessment of dietary habits over the past 6 mon prior to study entry. At T_3 and $T_{4.5}$ they recorded all food items and beverages consumed during the preceding 5 d. Intake of carotenoids was calculated using the food composition databases of Souci, Fachmann, and Kraut (13) and REGAL (14). Intake of energy, protein, fat, carbohydrates, and vitamins was calculated using the Austrian database Ernährungswissenschaftliches Programm (dato Denkwerkzeuge, Vienna, Austria, 1997).

Analytical methods. (i) Sample collection. At T_0, T_3, and $T_{4.5}$, venous blood was drawn after an overnight fast into plastic tubes containing either EDTA or lithium heparin (BD Vacutainer, Belliver Industrial Estate, Plymouth, United Kingdom), protected from light using aluminum foil, and centrifuged immediately at 1500 × *g* at 8°C for 10 min. Plasma was separated, divided into aliquots, collected in Eppendorf tubes, and stored at –80°C until determination of plasma carotenoids, retinol, tocopherols, and FA. Routine clinical chemistry indices, including total cholesterol, HDL, and TG, were determined in the fresh samples at T_0, T_3, and $T_{4.5}$ at the laboratory core facility of the University hospital (Clinical Institute of Medical and Chemical Laboratory Diagnostics) using a Hitachi analyzer (COPD test kit; Roche Diagnostics, Vienna, Austria). LDL was calculated using the formula of Friedewald *et al.* (15).

(ii) Determination of carotenoids, retinol, and tocopherols. The determination of retinol, tocopherols, and carotenoids was performed as described by Aebischer *et al.* (16). Briefly, 200 µL of EDTA plasma was diluted with deionized distilled water and deproteinized with 400 µL absolute ethanol. To extract lipophilic compounds, 800 µL of a solution of 350 mg BHT in 1000 mL *n*-hexane were added, centrifuged, and the clear supernatant transferred by a dispenser/dilutor system (Micro Laboratory 500B Dilutor; Hamilton, Martinsried, Germany) to an Eppendorf tube to be dried on a Speed Vac (Savant, Farmingdale, NY). The residue was then redissolved in a mixture of methanol/1,4-dioxane (1:1), diluted with acetonitrile, and injected into the HPLC system (Hewlett-Packard 1100A; Agilent, Vienna, Austria). Separation was achieved on a reversed-phase column; the mobile phase was a mixture of 684:220:68:28 acetonitrile/THF/methanol/water (by vol) containing 2.5 mg/L L(+)-ascorbate and 280 mg/L ammonium acetate, and the flow rate was 1.6 mL/min. α-Carotene, β-carotene, lutein/zeaxanthin, and β-cryptoxanthin were detected by a UV detector (JASCO, Model UV-1570; Biolab, Vienna, Austria) at 450 nm, and lycopene was detected at 472 nm; retinol and tocopherols were detected by a fluorescence detector (JASCO, Model FP-920; Biolab) at 330 nm excitation and 470 nm emission wavelength (retinol) and at 298 nm excitation and 328 nm emission wavelength (γ- and α-tocopherol). The areas under the HPLC peaks were quantified on an HP Chemstation (Hewlett-Packard 35900E; Agilent). *All-trans*- and *cis*-β-carotene were measured, and total β-carotene was calculated as the sum of *all-trans*- and *cis*-β-carotene. The tocopherol and carotenoid standards were a kind gift of the Vitamin Research Department of Hoffmann La-Roche (now DSM Nutritional Products), Basel, Switzerland. The CV within-run was 3.9% for retinol, 5.2% for γ-tocopherol, 5.0% for α-tocopherol, 5.7% for lutein/zeaxanthin, 5.4% for β-cryptoxanthin, 5.6% for lycopene, 4.7% for α-carotene, 6.0% for *cis*-β-carotene, and 5.1% for *all-trans*-β-carotene. All determinations were performed in duplicate, and all samples were processed in the same run. Six plasma samples of the patients were processed along with two control samples from a plasma pool obtained from healthy subjects to be used for long-term quality control, and along with a standard solution.

(iii) Determination of FA. The determination was based on an esterification procedure and a subsequent GC analysis of the FAME as described by Sattler *et al.* (17). Briefly, 100 µL of internal standard (heptadecanoic acid) was added to 450 µL of the EDTA plasma and kept at –80°C for a minimum of 30 min. The deep-frozen suspension was freeze-dried on a lyophilizer (VirTis, Gardiner, NY). Then, boron trifluoride/methanol complex and toluene were added. After transesterification at 110°C for 90 min, the FAME were extracted three times with *n*-hexane. The extracts

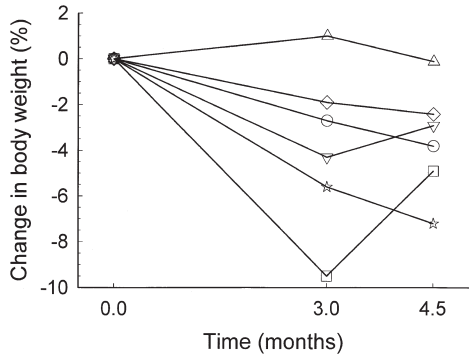

FIG. 1. Changes in body weight (% of baseline) in the individual patients during orlistat therapy.

were dried in a Speed Vac (Bachhofer, Reutlingen, Germany), redissolved in dichloromethane, and subjected to GC analysis (Hewlett-Packard 5890 Series II; Agilent). Separation of FAME was achieved on a DB-23 column, 30 m length, 0.250 mm diameter (Agilent); the mobile phase was a mixture of helium and hydrogen gas. The oven temperature at injection was 150°C and was then raised to 255°C. The areas under the GC peaks were quantified by integration, and the internal standard described above was used for calculation of the amounts of FA. CV (within-run) for the different FA were between 0.38 and 8.28%. All determinations were done in duplicate and in the same run, along with a control sample from the plasma pool.

Statistical analysis. All statistical analyses were performed on SigmaStat 3.0 (SPSS, Erkrath, Germany). Changes over time were analyzed using one-way repeated measures ANOVA. To isolate the time points that differed from the others, all-pairwise multiple comparison procedures (Tukey tests) were used.

RESULTS

Effects of intervention. All study subjects completed the 4.5-mon study period. Compliance with orlistat therapy was 100%, as checked by interview and pill count. One patient reported steatorrhea and diarrhea after she had not adhered to the dietary advice of reduced fat intake. Additional side effects were not reported.

Anthropometry. Changes in body weight showed substantial interindividual variability. While all patients except one showed a significant weight loss during the initial 3 mon, only four of six lost weight during the following 1.5-mon of therapy (Fig. 1). Overall, changes in weight, BMI, body fat, and hip and waist circumference were not statistically significant (Table 1). Body fat could not be measured in one patient due to problems with the technical equipment (BIA analyzer; Eumetics) at T_3 and in another one due to phlebitis at $T_{4.5}$.

Dietary assessment. The study subjects showed a high percentage of energy intake from fat at T_3 (38.5 ± 5.95%) and at $T_{4.5}$ (37.3 ± 5.19%). The average daily energy intake was 7729 ± 1639 kJ at T_3 and 8327 ± 2194 kJ at $T_{4.5}$. Dietary intakes of carotenoids calculated from FFQ at T_0 and from 5-d food records at T_3 and $T_{4.5}$ are presented in Table 2. There were no changes in dietary intakes of the different carotenoids during orlistat therapy, but wide ranges of consumed fruits and vegetables among the patients were observed (Table 2). There was no significant correlation between weight loss and energy intake ($P = 0.78$ at T_3 and $P = 0.26$ at $T_{4.5}$).

TABLE 2
Dietary Intake[a] (mean ± SD) of Carotenoids (mg/d) and Fruits and Vegetable Servings (servings/d) Estimated by FFQ (T_0) and 5-d Food Records (T_3 and $T_{4.5}$) ($n = 6$)

	FFQ	5-d food records	
	T_0	T_3	$T_{4.5}$
α-Carotene	0.487 ± 0.298	0.671 ± 0.734	0.959 ± 0.881
β-Carotene	3.77 ± 2.56	3.11 ± 2.23	3.73 ± 3.40
Lycopene	3.50 ± 2.65	1.69 ± 1.25	1.38 ± 1.56
β-Cryptoxanthin	0.492 ± 0.296	0.587 ± 0.663	0.517 ± 0.868
Lutein/zeaxanthin	4.58 ± 3.91	2.64 ± 1.51	1.77 ± 1.42
Servings of fruits and vegetables per day	5.20 ± 3.61	2.77 ± 1.88	2.60 ± 1.37

[a]There were no significant differences between T_3 and $T_{4.5}$ for any of the variables studied. FFQ, food frequency questionnaire.

TABLE 3
Changes in Plasma Carotenoid Concentrations (mean ± SD, μmol/L) During Orlistat Therapy ($n = 6$)

	Time points			
	T_0	T_3	$T_{4.5}$	P value[a]
Raw values				
α-Carotene	0.106 ± 0.058[b]	0.081 ± 0.052	0.058 ± 0.043[b]	0.006
β-Carotene	0.543 ± 0.310[c,d]	0.392 ± 0.347[c]	0.369 ± 0.227[d]	0.013
Lycopene	0.362 ± 0.371	0.184 ± 0.272[e]	0.338 ± 0.391[f]	0.004
β-Cryptoxanthin	0.275 ± 0.123	0.215 ± 0.124	0.267 ± 0.183	NS
Lutein/zeaxanthin	0.343 ± 0.046	0.305 ± 0.091	0.341 ± 0.182	NS
Standardized values				
α-Carotene: cholesterol	0.015 ± 0.009[g]	0.012 ± 0.008	0.008 ± 0.006[g]	0.016
β-Carotene: cholesterol	0.072 ± 0.043[h]	0.055 ± 0.047	0.051 ± 0.030[h]	0.030
Lycopene: cholesterol	0.048 ± 0.050[i]	0.025 ± 0.037[i,j]	0.046 ± 0.051[j]	0.005
β-Cryptoxanthin: cholesterol	0.036 ± 0.018	0.031 ± 0.018	0.037 ± 0.024	NS
Lutein/zeaxanthin: cholesterol	0.045 ± 0.005	0.043 ± 0.013	0.047 ± 0.025	NS

[a]Repeated measures ANOVA. [b–j]Significant differences by Tukey *post-hoc* tests are indicated with similar superscripts. NS, not significantly different.

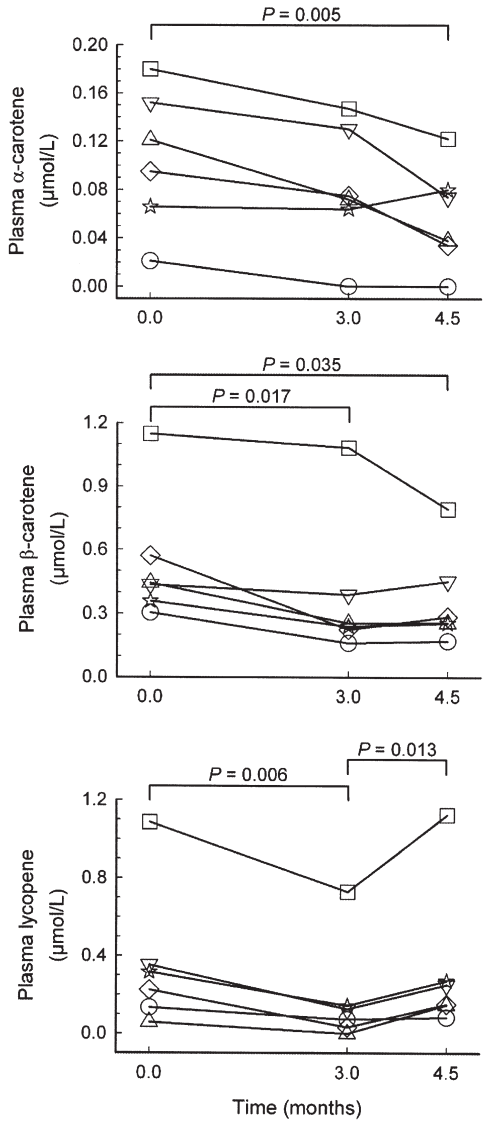

FIG. 2. Changes in plasma concentrations of α- and β-carotene and lycopene in the individual patients during orlistat therapy. *P* values were obtained by repeated measures ANOVA and Tukey all-pairwise multiple comparison procedures (for details see text and Table 3). Similar symbols indicate similar subjects.

Plasma carotenoids. Mean plasma concentrations and standardization for cholesterol of the different carotenoids at the three time points are presented in Table 3, and changes in plasma carotenoid concentrations of the individual patients are presented in Figure 2. Plasma α-carotene, β-carotene, and lycopene concentrations showed significant changes across the three time points (Fig. 2) (one-way repeated measures ANOVA), whereas β-cryptoxanthin and lutein/zeaxanthin did not. The Tukey all-pairwise multiple comparison procedure showed a significant decrease for α-carotene from T_0 to $T_{4.5}$ ($P = 0.005$), and for β-carotene from T_0 to T_3 ($P = 0.017$) and T_0 to $T_{4.5}$ ($P = 0.035$). Plasma lycopene concentrations decreased significantly from T_0 to T_3 ($P = 0.006$), and increased again from T_3 to $T_{4.5}$ ($P = 0.013$). Similar findings were observed for carotenoids standardized for cholesterol (Table 3). There were significant correlations between the carotenoid intakes and plasma concentrations for β-carotene at T_3 ($P = 0.04$), for lycopene at $T_{4.5}$ ($P = 0.02$), and for β-cryptoxanthin at T_3 ($P = 0.02$).

Serum lipids, plasma fat-soluble vitamins, and FA. Changes in total cholesterol (7.77 ± 1.18 at T_0, 7.15 ± 0.969 at T_3, and 7.23 ± 0.492 at $T_{4.5}$), HDL-cholesterol (1.45 ± 0.385 at T_0, 1.42 ± 0.258 at T_3, and 1.44 ± 0.395 at $T_{4.5}$), LDL-cholesterol (5.03 ± 0.705 at T_0, 4.86 ± 1.20 at T_3, and 4.64 ± 0.490 at $T_{4.5}$), and TG (2.84 ± 1.42 at T_0, 2.89 ± 1.93 at T_3, and 2.53 ± 0.991 at $T_{4.5}$) were not significant. Changes in plasma retinol (2.41 ± 1.13 at T_0, 2.51 ± 0.801 at T_3, and 2.29 ± 0.756 at $T_{4.5}$), α-tocopherol (37.2 ± 8.66 at T_0, 37.6 ± 4.54 at T_3, and 36.4 ± 6.15 at $T_{4.5}$) and γ-tocopherol (3.53 ± 2.27 at T_0, 3.01 ± 1.10 at T_3, and 2.62 ± 1.12 at $T_{4.5}$) were also not significant. In addition, plasma FA concentrations did not change significantly.

DISCUSSION

This is the first study showing plasma concentrations of the different hydrocarbon and oxygenated carotenoids during orlistat therapy and therefore allowing for comparison of the effects of orlistat based on the chemical structure of carotenoids.

Plasma α-carotene, β-carotene, and lycopene concentrations showed significant changes across the three time points, whereas β-cryptoxanthin and lutein/zeaxanthin did not. These results indicate that plasma concentrations of the hydrocarbon carotenoids α- and β-carotene and lycopene are more severely affected by orlistat-induced inhibition of gastrointestinal lipase activity than those of the less hydrophobic oxygenated carotenoids β-cryptoxanthin and lutein/zeaxanthin. The decrease in plasma α-carotene concentrations by 45% to 0.058 μmol/L and in plasma β-carotene by 32% to 0.369 μmol/L resulted in plasma concentrations that were about 76 and 60% lower for α-carotene and 59 and 43% lower for β-carotene compared with those of healthy Swiss (18) and Austrian subjects (19), respectively. In contrast, plasma concentrations of α- and β-carotene were comparable whereas plasma lycopene concentrations were lower during orlistat therapy in our study compared with serum concentrations of healthy subjects participating in the NHANES III (Third National Health and Nutrition Examination Survey) study (20). It seems unlikely that the changes in plasma concentrations in the present study were due to changes in intakes of α- and β-carotene, given that dietary recommendations focused on high intakes of fruits and vegetables, which are associated with higher intake of carotenoids. Unfortunately, prospective dietary records, in addition to FFQ, were not available at baseline to allow for assessment of prospective dietary intake data. There was a substantial difference in the consumption of fruits and vegetables recorded by FFQ and 5-d food records, respectively, suggesting that intakes might have been overestimated in FFQ.

Intestinal absorption of fat-soluble compounds such as carotenoids is facilitated when ingested along with dietary lipids (11). The study subjects showed a high percentage of energy intake from fat, which contrasted the recommended dietary regimen but is expected to have facilitated efficient absorption of

lipophilic compounds. In the absence of changes in dietary habits and lack of significant weight loss, the decrease in α- and β-carotene concentrations observed in our study can be explained by impaired lipase activity and subsequently reduced intestinal absorption during orlistat therapy. The significance of intestinal absorption of dietary lipids to facilitate specifically the absorption of carotenoids is further underlined by a recent study showing decreased plasma concentrations of carotenoids when olestra, an indigestible sucrose polyester containing 6–8 FA per molecule, was used to replace one-third of energy from fat. This effect could not be prevented by carotenoid supplements (21).

In the present 4.5-mon long-term study, a one-third reduction of β-carotene concentrations was observed during 360 mg/d orlistat therapy. These results are in agreement with a 6-mon long-term study showing a 22–40% reduction by using 90–720 mg/d orlistat (22). In contrast, 180 and 360 mg/d orlistat in a 2-yr long-term study did not show a significant effect (23).

Plasma concentrations of retinol were within the normal range and did not change during orlistat therapy. Both single-dosing (5,24) and long-term treatment with orlistat (22–25) did not alter plasma retinol concentrations, which can be explained by homeostatic control of plasma retinol concentrations.

The decrease in plasma α- and γ-tocopherol concentrations in the present 4.5-mon study was not significant. Other groups found impaired absorption of α-tocopherol taken along with orlistat in a single-dose test situation (5,24), whereas long-term treatment with orlistat did not alter plasma tocopherol concentrations (24,25).

In the present study, 4.5-mon orlistat therapy was not associated with statistically significant changes in plasma FA patterns, in contrast to 12-mon orlistat therapy in another study on 75 patients (7). There were also no significant changes in serum lipids during orlistat therapy. Although the cholesterol- and TG-lowering effect was smaller in our study compared with some other orlistat trials (23,26), it was comparable to those obtained in randomized placebo-controlled trials (7,27) that showed a decrease in plasma lipids that did not reach statistical significance. However, it cannot be ruled out that the findings of the present study could be due to the small number of study subjects (possible type II error), and extension of the study period might have allowed for significant changes to occur in FA patterns.

Taken together, significant changes in α- and β-carotene as well as in lycopene were observed even in the presence of relatively low weight losses. The latter were 0.1–7.2% (3.6 ± 2.4%) over 4.5 mon of orlistat therapy in the study patients compared with weight losses of 6.8–9.8% over 6 mon in other trials (20,26,28). However, there was a wide range in weight losses in the present as well as in previous studies (20,23,26,28), suggesting that some patients respond more favorably than others to orlistat (29).

Epidemiological evidence suggests that high intake of carotenoids and plasma concentrations in the upper normal range are associated with lower risk of cardiovascular disease and certain types of cancer (12). A recent study demonstrated that plasma α-carotene and γ-tocopherol concentrations are lower in patients with coronary heart disease compared with healthy controls (30). Given the preventive effects of carotenoids, a decrease in plasma concentrations during orlistat therapy may add an additional risk factor for obese patients, who are at increased risk for degenerative diseases. This seems even more important in view of the fact that the study patients showed low carotenoid status already prior to orlistat therapy, which, at least in part, can be explained by unfavorable dietary habits. Dietary intake of both α- and β-carotene was considerably lower in our study patients than in nonobese healthy male subjects living in the same area (19). This is even more striking because with one exception the study patients were females, who, in general, consume a diet higher in carotenoids compared with males (31).

It has been suggested that plasma concentrations of fat-soluble vitamins should be monitored and multivitamin supplements should be taken during orlistat therapy (20,22–25). However, high intake of carotenoids from fruits and vegetables during orlistat therapy might prove even more beneficial and contribute to the overall success of orlistat therapy, given that it would avoid possible unwanted effects of β-carotene supplements on the one hand (32), and facilitate lower total calorie and fat intake on the other.

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Isabella Sundl developed the study design under the supervision of Brigitte M. Winklhofer-Roob and Hermann Toplak. Elisabeth Pail, Karin Mellitzer, and Hermann Toplak recruited and investigated the patients and collected the blood samples. Isabella Sundl carried out the analyses of the blood samples and the dietary records and performed the statistical analysis under the supervision of Brigitte M. Winklhofer-Roob. Isabella Sundl and Brigitte M. Winklhofer-Roob had primary responsibility for writing the manuscript, but all the other authors provided editorial comments on the draft. None of the authors had any conflict of interest.

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Positive Association Between Plasma Antioxidant Capacity and n-3 PUFA in Red Blood Cells from Women

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ABSTRACT: PUFA are susceptible to oxidation. However, the chain-reaction of lipid peroxidation can be interrupted by antioxidants. Whether an increased concentration of PUFA in the body leads to decreased antioxidant capacity and/or increased consumption of antioxidants is not known. To elucidate the relationship between plasma total antioxidant capacity (TAC), the concentration of antioxidant vitamins, and the proportion of PUFA in red blood cells (RBC), plasma TAC was measured by a Trolox equivalent antioxidant capacity assay in blood samples from 99 Icelandic women. Concentrations of tocopherols and carotenoids in the plasma were determined by HPLC, and the FA composition of RBC total lipids was analyzed by GC. Plasma TAC and the plasma concentration of α-tocopherol correlated positively with the proportion of total n-3 PUFA, 20:5n-3, and 22:6n-3 in RBC, whereas the plasma lycopene concentration correlated negatively with the proportion of total n-3 PUFA and 20:5n-3. On the other hand, plasma TAC correlated negatively with the proportion of n-6 PUFA in RBC. Plasma TAC also correlated positively with the plasma concentration of α-tocopherol, alcohol consumption, and age. Both the plasma concentration of α-tocopherol and age correlated positively with the proportion of n-3 PUFA in RBC; however, n-3 PUFA contributed independently to the correlation with plasma TAC. Because the proportion of n-3 PUFA in RBC reflects the consumption of n-3 PUFA, these results suggest that dietary n-3 PUFA do not have adverse effects on plasma TAC or the plasma concentration of most antioxidant vitamins.

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PUFA are prone to oxidation, with increased susceptibility to oxidation being linked to the degree of unsaturation (1). The more unsaturated n-3 PUFA, EPA (20:5n-3) and DHA (22:6n-3), are thought to be more susceptible to oxidation than the n-6 PUFA arachidonic acid (AA, 20:4n-6), but recent findings also suggest that n-3 PUFA may decrease or protect against oxidative stress (2,3). Several human studies show that increased consumption of n-3 PUFA or fish oil leads to increased oxidation. For example, healthy smokers receiving 4 g of fish oil supplements per day for 3 wk had increased oxidation of plasma LDL (4), and long-term intervention with high doses of n-3 PUFA (850–882 mg EPA or DHA) for 1 yr, following an acute myocardial infarction, increased lipid peroxidation, measured as the thiobarbituric acid–malondialdehyde complex (5). Other studies show no effect of n-3 PUFA, or fish oil, on various indices of oxidation. For example, there was no change in the resistance of red blood cells (RBC) to hemolysis in normo- and hypertriglyceridemic subjects receiving 6 g of fish oil per day for 6 wk (6) or in plasma malondialdehyde in ulcerative colitis patients receiving 4.5 g of n-3 PUFA per day for 2 mon (3). In the latter study, fish oil n-3 PUFA decreased oxidation, as assessed by plasma chemiluminescence (3), and in another recent study, EPA decreased oxidative stress, as measured by urinary 8-isoprostane levels, in patients with lupus nephritis (2). Discrepancies among results from studies evaluating the effects of fish oil or n-3 PUFA on oxidation may be related to the different methods used to assess oxidation as well as the amount of n-3 PUFA in the diet (6–8), the duration of treatment, whether the subjects were under oxidative stress (3) or not (9), and lifestyle factors such as smoking (10) and drinking (11).

The body stores antioxidants that can be mobilized to neutralize reactive oxygen species and that are important for interrupting the chain reaction of lipid peroxidation. Scavenging antioxidants, such as α-tocopherol and β-carotene, have a high redox potential but are consumed in the process of oxidation. That increased PUFA consumption may decrease antioxidant status was demonstrated by the classic means of inducing vitamin E deficiency in animals, which is by feeding fish oil (12). Lower plasma vitamin E levels were also seen in women after receiving fish oil supplements containing 1.680 mg EPA, 720 mg DHA, and 6 IU of vitamin E for 3 mon (13) and in healthy men after receiving fish oil concentrate (15 g/d) fortified with 15 mg vitamin E for 10 wk (14). However, daily supplementation with 2.5 g EPA and 1.8 g DHA, without added vitamin E, for 5 wk had no effect on plasma α- and γ-tocopherol levels in healthy women (15). Further, supplementation of 2.1 g of fish oil, without added vitamin E, for 1 mon increased the plasma concentrations of α-tocopherol and β-carotene in healthy women (7).

Results from studies evaluating the effect of n-3 PUFA on antioxidant capacity differ. Animal studies show both an increase (16) and a decrease (17) in plasma total antioxidant capacity (TAC) following fish oil feeding. When healthy humans

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Abbreviations: AA, arachidonic acid; $ABTS^{\bullet+}$, 2,2´-azinobis (3-ethylbenzothiazoline) radical cation; PI, peroxidizability index; RBC, red blood cells; TAC, total antioxidant capacity; TIBC, total iron-binding capacity.

supplemented their diets with fish oil and vitamin E, it had no effect on plasma TAC (18). Neither was there an effect of long-term DHA supplementation on plasma TAC in patients with X-linked retinitis pigmentosa (19). In contrast, treatment with fish oil n-3 PUFA increased microsomal lipid peroxidation inhibition in patients with lupus nephritis (3), and a negative correlation was found between antioxidant capacity, measured as anti-radical defense capacity against hemolysis in whole blood, and low fish consumption in healthy humans (20).

From these studies, there is no clear indication of whether dietary n-3 PUFA increase oxidation, decrease the plasma concentration of antioxidants or affect the antioxidant capacity in the body. Thus, the present study was performed to evaluate whether the proportion of PUFA in RBC from Icelandic women, who exhibit a wide range in proportions of n-3 PUFA in the RBC, was correlated with plasma TAC and antioxidant vitamin concentration, taking into account several potential confounding factors.

SUBJECTS AND METHODS

Subjects. Participants in this study were 99 female relatives of cancer patients enrolled in a larger study on genetic and environmental risk factors in breast cancer performed by the Icelandic Cancer Society. All women enrolled in the larger study at the time of recruitment for participation in the present study (March 2001–March 2003) were invited to participate in the present one. All participants signed an informed consent form prior to enrollment in the study. The study was approved by the National Bioethics Committee in Iceland (99–084-B1-S1), and the Data Protection Authority in Iceland was notified of the processing of personal data (216/2001). Participants donated blood after an overnight fast. Information on putative confounding factors (smoking habits, alcohol intake, fish liver oil intake, body weight and height) was obtained by a standardized lifestyle questionnaire. Smoking habits were recorded as cigarettes smoked per day. Alcohol consumption was recorded as grams of ethanol per day, where 10 g of ethanol equaled one bottle of beer (330 mL), one glass of white or red wine (125 mL), one drink of liquor (30 mL), or one drink of spirits (30 mL). Continuous fish oil supplement intake for the past 2 yr was recorded. Fish liver oil was not specified according to origin.

Plasma TAC. TAC was measured in EDTA plasma using a photometric method described by Rice-Evans and Miller (21). Plasma samples were prepared immediately after blood collection, flushed with nitrogen, and stored at –70°C until analyzed within 3 wk. The method measures the inhibitory effect of plasma antioxidants on the absorbance of 2,2´-azinobis (3-ethylbenzothiazoline) radical cation ($ABTS^{\bullet+}$) generated by activation of metmyoglobin (myoglobin equine heart; Sigma-Aldrich, Steinheim, Germany) with H_2O_2. Trolox (Fluka Chemie, Buchs, Switzerland), a water-soluble analog of α-tocopherol, was used as a standard, and units of activity were expressed as mmol/L of Trolox equivalents. Each plasma sample was measured in duplicate. The interassay CV was 3.0%.

Plasma tocopherols and carotenoids. Tocopherols and carotenoids were measured in EDTA plasma flushed with nitrogen and stored at –70°C until analysis was performed. Aliquots were rapidly thawed at room temperature and were protected from light. Plasma concentrations of α- and γ-tocopherol, α- and β-carotene, β-cryptoxanthin, and lycopene were determined by RP-HPLC (HPLC system series 200, Perkin-Elmer) using a method described by Su *et al.* (22). Samples were extracted with hexane after precipitating the proteins with ethanol, then evaporated under nitrogen and redissolved in chloroform. After vortexing for 40 s and adding acetonitrile/methanol (1:1, vol/vol), samples were vortexed for a further 40 s. The mobile phase consisted of methanol with 0.05% ammonium acetate, acetonitrile with 0.1% triethylamine, and chloroform. Three linear gradient steps were programmed. Samples were injected onto a reversed-phase C18 5-μm particle diameter column. α- and γ-Tocopherols were monitored at 292 nm and α- and β-carotene, β-cryptoxanthin, and lycopene at 450 nm. Ratios of peak areas of the samples to peak areas of an internal standard were compared with those of a certified serum standard (standard reference material 968c; NIST, Gaithersburg, MD). Each sample was analyzed in duplicate. The interassay CV was 3.4% for α-tocopherol, 4.1% for γ-tocopherol, 18.1% for α-carotene, 9.1% for β-carotene, 3.8% for β-cryptoxanthin, and 6.6% for lycopene.

Body iron status. Serum iron was measured with the ferrozine dye-binding method (Roche Iron kit; Roche Diagnostics, Mannheim, Germany). Total iron-binding capacity (TIBC) in serum was measured as the sum of measured serum iron and unsaturated iron-binding capacity of transferrin (Roche Diagnostics) or indirectly with a transferrin-immunoassay (Transferrin ver. 2; Roche Diagnostics). Serum transferrin saturation was calculated as serum iron/serum TIBC × 100. Serum ferritin was measured with an electrochemiluminescence immunoassay (Elecsys Ferritin reagent kit; Roche Diagnostics).

Lipid extraction and FA analysis of RBC. RBC were isolated on Histopaque from venous blood and washed three times with saline. RBC, in the presence of the antioxidant BHT (20 mg/L) and nitrogen, were kept at –70°C until analysis. Total lipids where extracted from RBC using chloroform/isopropanol as described by Rose and Oklander (23) with some modifications. Briefly, total lipids of RBC were extracted with chloroform/isopropanol (1:2, vo/vol), and BHT (5 mg/100 mL) was added to the extraction medium. The FA of RBC total lipids were methylated for 45 min at 110°C using 14% boron trifluoride/methanol (Merck, Hohenbrun, Germany). The FAME were analyzed using GC (Hewlett-Packard HP 7673; Hewlett-Packard, Palo Alto, CA) equipped with a Chrompack CP-Sil 8CB PEG column (30 m × 250 μm i.d. × 0.25 μm film thickness). The oven temperature was programmed to have an initial oven temperature of 150°C for 4 min, then rising at 4°C/min to 230°C and at 20°C/min to 280°C, then isothermal for 4 min. The injector and detector temperatures were maintained at 280 and 300°C, respectively. Hydrogen was used as the carrier gas. The FAME peaks were identified and calibrated against those of commercial standards (Sigma, St. Louis, MO; Nu-Chek-Prep, Elysian, MN). PC diheptadecanoyl (17:0) and heneicosanoic acid (21:0) methyl ester (Sigma) were used as internal and external standards,

respectively. The results are expressed as g/100 g of total FA. Each sample was analyzed in duplicate.

Statistical analysis. Mean levels of plasma TAC were compared between groups (smokers/nonsmokers, fish oil intake/no fish oil intake) using unpaired *t*-tests, and the results are expressed as mean values ± SD. In univariate analyses, the association between variables was evaluated using Pearson's correlation coefficient (*r*). Multiple regression modeling was applied to evaluate the association between PUFA and TAC, taking into account the effects of potential confounding factors. The significance level was set at $P < 0.05$. The statistical analysis was performed with SPSS 11.5 for Windows.

RESULTS

The associations among plasma TAC, plasma concentration of antioxidant vitamins, iron status, and proportions of FA in RBC from Icelandic women were examined, and potential confounding factors were taken into account. Characteristics of the subject group are summarized in Table 1. Mean levels of plasma TAC, concentrations of plasma tocopherols and carotenoids, and iron status are shown in Table 2. Women consuming fish oil supplements continually for 2 yr ($n = 37$) did not differ in plasma TAC (1.1 ± 0.1 mmol/L vs. 1.1 ± 0.2 mmol/L, respectively) or indices of iron status (data not shown) from women not consuming fish oil supplements ($n = 62$), but had higher plasma concentrations of α-tocopherol (29 ± 10 μmol/L vs. 25 ± 7 μmol/L, respectively, $P < 0.05$) and lower plasma concentrations of γ-tocopherol (2.7 ± 0.8 μmol/L vs. 3.1 ± 1.0 μmol/L, respectively, $P < 0.05$). There was no difference in plasma TAC, plasma concentrations of most of the antioxidant vitamins, or indices of iron status between smokers ($n = 24$) and nonsmokers ($n = 75$). Only the plasma concentration of β-cryptoxanthin was lower in smokers than in nonsmokers (0.08 ± 0.08 μmol/L vs. 0.14 ± 0.12 μmol/L, respectively, $P < 0.05$) and correlated negatively with the number of cigarettes smoked per day ($r = –0.200$, $P < 0.05$).

The FA composition and peroxidizability index (PI) of RBC total lipids are shown in Table 3. Women consuming fish oil supplements continually for 2 yr had a higher proportion of n-3 PUFA in the RBC than women not consuming fish oil supplements (13.1 ± 2.0 g/100 g vs. 11.3 ± 1.4 g/100 g, respectively, $P < 0.0001$). Women who smoked had a lower proportion of n-3 PUFA in the RBC than did women who did not smoke (11.16 ± 0.32 g/100 g vs. 12.17 ± 0.22 g/100 g, respectively, $P < 0.05$).

TABLE 1
Characteristics of Participants

	n	Mean ± SD	Range
Age (yr)	99	45.8 ± 12.1	18–73
Weight (kg)	98	71.9 ± 12.5	51–118
Height (cm)	99	167.7 ± 6.2	155–184
BMI[a] (kg/m^2)	98	25.6 ± 4.0	18.1–39.4
Smoking (cigarettes/d)	24	9.6 ± 6.7	2–30
Alcohol (g/d)	77	3.4 ± 3.8	0.2–20

[a]BMI, body mass index.

TABLE 2
Plasma Total Antioxidant Capacity (TAC), Concentrations of Plasma Tocopherols and Carotenoids, Serum Concentration of Iron, Total Iron-Binding Capacity (TIBC), Transferrin Saturation, and Ferritin in Samples from 99 Icelandic Women

	Mean ± SD
TAC (mmol/L)	1.10 ± 0.16
α-Tocopherol (μmol/L)	26.94 ± 8.78
γ-Tocopherol (μmol/L)	2.97 ± 0.97
α-Carotene (μmol/L)	0.08 ± 0.12
β-Carotene (μmol/L)	0.35 ± 0.37
β-Cryptoxanthin (μmol/L)	0.13 ± 0.11
Lycopene/(μmol/L)	0.59 ± 0.43
Iron (μmol/L)	16.4 ± 7.7
TIBC (μmol/L)	65.9 ± 13.7
Transferrin saturation (%)	25.8 ± 11.8
Ferritin (μg/L)	46.3 ± 41.1

Correlations between plasma TAC and plasma concentrations of antioxidant vitamins and the proportion of FA in RBC are shown in Table 4. Plasma TAC correlated positively with the proportions of total n-3 PUFA (Fig. 1), 20:5n-3, and 22:6n-3 in RBC (Table 4). The plasma concentration of α-tocopherol also correlated positively with the proportions of total n-3 PUFA, 20:5n-3, and 22:6n-3 in RBC, whereas the plasma concentration of lycopene correlated negatively with the proportions of total n-3 PUFA and 20:5n-3 (Table 4). Analysis of population quartiles revealed a difference in plasma TAC between the subpopulations with the lowest ($P < 0.05$) and the second lowest ($P < 0.05$) proportions of n-3 PUFA in RBC and the subpopulation with the highest proportion of n-3 PUFA in RBC (1st quartile, 1.04 ± 0.18 mmol/L; 2nd quartile, 1.03 ± 0.14

TABLE 3
FA Composition (g/100 g of total lipids) and Peroxidizability Index (PI) in Red Blood Cell (RBC) Total Lipids from 99 Icelandic Women

FA[a]	Mean ± SD
Total SFA	40.73 ± 1.01
16:0	20.17 ± 0.96
18:0	15.25 ± 0.77
22:0	1.21 ± 0.20
24:0	3.21 ± 0.35
Total MUFA	15.80 ± 1.19
18:1n-9	12.08 ± 0.87
24:1n-9	2.95 ± 0.50
Total PUFA	38.97 ± 1.32
Total n-6 PUFA	26.74 ± 2.57
18:2n-6	10.9 ± 1.28
20:3n-6	1.63 ± 0.36
20:4n-6	13.02 ± 1.56
22:4n-6	2.25 ± 0.63
Total n-3 PUFA	11.92 ± 1.86
20:5n-3	1.45 ± 0.73
22:5n-3	3.16 ± 0.37
22:6n-3	7.31 ± 1.10
PI[b]	111.3 ± 4.9

[a]FA that were less than 1% of total FA in RBC are excluded. SFA, saturated FA; MUFA, monounsaturated FA.
[b]PI: (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 3) + (% pentaenoic × 4) + (% hexaenoic × 5).

TABLE 4
Correlation Between Plasma TAC (mmol/L) and Plasma Concentrations of Antioxidant Vitamins (μmol/L) and the Proportion of FA (g/100 g) in RBC Total Lipids from 99 Icelandic Women (r^a and *P* value)

FA	TAC	α-Tocopherol	γ-Tocopherol	α-Carotene	β-Carotene	β-Cryptoxanthin	Lycopene
Total SFA	0.066	–0.040	0.041	–0.104	–0.162	0.055	–0.061
	0.519	0.697	0.689	0.307	0.109	0.589	0.549
Total MUFA	0.177	0.162	–0.033	0.197	0.216	–0.007	–0.042
	0.080	0.110	0.745	0.051	0.032[b]	0.941	0.683
Total PUFA	0.160	–0.016	0.097	–0.165	–0.108	0.009	0.058
	0.113	0.874	0.339	0.102	0.288	0.927	0.568
Total n-6 PUFA	–0.243	–0.183	0.107	–0.060	–0.204	–0.013	0.248
	0.015[b]	0.070	0.290	0.553	0.042[b]	0.895	0.013[b]
18:2n-6	–0.195	–0.011	0.178	–0.052	0.023	0.057	0.141
	0.054	0.910	0.079	0.609	0.822	0.577	0.164
20:4n-6	–0.259	–0.207	0.037	–0.003	–0.107	–0.115	0.094
	0.010[b]	0.039[b]	0.719	0.975	0.290	0.258	0.357
22:4n-6	–0.259	–0.232	0.005	–0.111	–0.226	–0.092	0.201
	0.010[b]	0.021[b]	0.964	0.276	0.024[b]	0.366	0.046[b]
Total n-3 PUFA	0.347	0.255	–0.103	–0.008	0.108	0.166	–0.205
	<0.001[b]	0.011[b]	0.311	0.935	0.288	0.101	0.042[b]
20:5n-3	0.339	0.266	–0.015	0.031	0.136	0.112	–0.213
	0.001[b]	0.008[b]	0.882	0.760	0.178	0.269	0.035[b]
22:6n-3	0.315	0.244	–0.195	–0.065	0.035	0.175	–0.142
	0.002[b]	0.015[b]	0.053	0.523	0.728	0.083	0.161

[a]Pearson's correlation coefficient (*r*).
[b]Correlation between variables statistically significant. For abbreviations see Tables 2 and 3.

mmol/L; 3rd quartile, 1.14 mmol/L; 4th quartile 1.17 ± 0.17 mmol/L).

There was a negative correlation between plasma TAC and the proportions of total n-6 PUFA, 20:4n-6, and 22:4n-6 in RBC (Table 4). In a multivariate analysis including both the proportions of n-3 and n-6 PUFA in RBC as independent variables, only the proportion of n-3 PUFA in RBC showed an independent contribution to the association with plasma TAC.

Plasma TAC and the concentration of α-tocopherol correlated positively with age ($r = 0.296$, $P < 0.005$, and $r = 0.396$, $P < 0.0001$, respectively), whereas the plasma concentration of lycopene correlated negatively with age ($r = -0.437$, $P < 0.0001$). The proportions of total n-3 PUFA (Fig. 2), 20:5n-3, and 22:6n-3 in RBC correlated positively with age ($r = 0.539$, $P < 0.0001$; $r = 0.551$, $P < 0.001$; and $r = 0.438$, $P < 0.0001$, respectively). In a multivariate analysis with age and the proportion of total n-3 PUFA in RBC as independent variables, only the proportion of n-3 PUFA in RBC showed an independent contribution to the association with plasma TAC.

There was a positive correlation between plasma TAC and alcohol consumption ($r = 0.328$, $P < 0.005$). There was also a positive correlation between plasma concentrations of both α-tocopherol and lycopene and alcohol consumption ($r = 0.319$, $P < 0.005$, and $r = 0.275$, $P < 0.05$, respectively). In addition, there was a positive correlation between the proportions of n-3 PUFA in RBC and alcohol consumption ($r = 0.45$, $P < 0.001$). Because plasma TAC correlated positively with both the proportion of total n-3 PUFA in RBC and alcohol consumption, we examined whether either or both of these variables were independent contributors to the association with plasma TAC. In a multivariate model, with the proportion of n-3 PUFA in RBC and alcohol consumption as independent variables, neither variable showed a significant independent contribution to the model, although the overall relationship with plasma TAC was statistically significant ($r = 0.374$, $P < 0.005$). However, when the analysis was limited to women who consumed less than 6 g of ethanol per day (68 of 79 participants who responded to the question about alcohol intake), there was a positive correlation between plasma TAC and the proportion of n-3 PUFA in RBC ($r = 0.257$, $P < 0.05$), but there was no correlation between plasma TAC and alcohol consumption (data not shown).

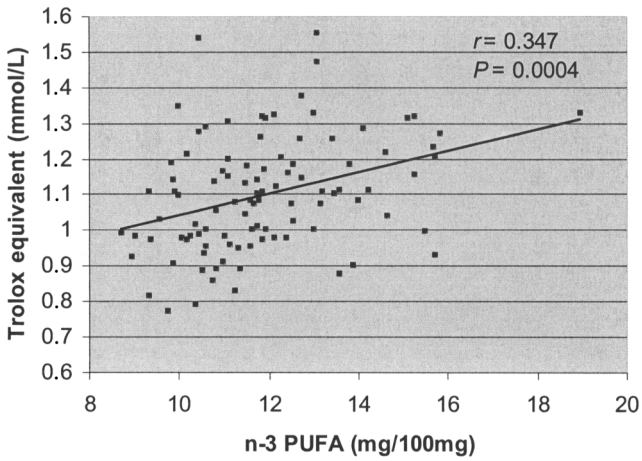

FIG. 1. Association between plasma total antioxidant capacity (TAC) and the proportion of total n-3 PUFA in red blood cell (RBC) total lipids from 99 Icelandic women. The association was evaluated using Pearson's correlation coefficient (*r*). Each sample was measured in duplicate.

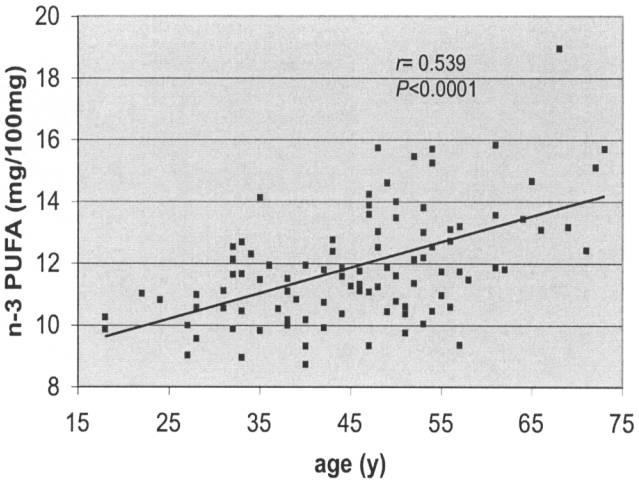

FIG. 2. Association between the proportion of total n-3 PUFA in RBC total lipids and age (yr) of 99 Icelandic women. The association was evaluated using Pearson's correlation coefficient (*r*). Each sample was measured in duplicate. For abbreviation see Figure 1.

There was a positive correlation between plasma TAC and both the plasma concentration of α-tocopherol and serum ferritin (Table 5). In a multivariate analysis with the concentration of ferritin in serum, the concentration of α-tocopherol in plasma, and the proportion of n-3 PUFA in RBC, only n-3 PUFA showed an independent contribution to the association with plasma TAC.

TABLE 5
Correlation Between Plasma TAC and Concentrations of Plasma Tocopherols and Carotenoids, Concentration of Serum Iron and Ferritin, and TIBC in Samples from 99 Icelandic Women

	r^a	*P* value
α-Tocopherol (μmol/L)	0.341	0.001[b]
γ-Tocopherol (μmol/L)	0.146	0.150
α-Carotene (μmol/L)	0.026	0.799
β-Carotene (μmol/L)	–0.017	0.869
β-Cryptoxanthin (μmol/L)	0.057	0.574
Lycopene (μmol/L)	–0.024	0.814
Iron (μmol/L)	0.097	0.344
TIBC (μmol/L)	0.103	0.311
Transferrin saturation (%)	0.033	0.747
Ferritin (μg/L)	0.265	0.010[b]

[a]Pearson's correlation coefficient.
[b]Correlation between variables statistically significant. For abbreviations see Table 2.

DISCUSSION

Because n-3 PUFA are highly unsaturated and believed to be prone to oxidation, they may lead to lower antioxidant capacity and increased consumption of antioxidants in the body. The only indication in the current study of increased consumption of antioxidants in the body with increased n-3 PUFA was the negative correlation between plasma lycopene concentration and the proportion of n-3 PUFA in RBC. On the other hand, the positive correlation between both plasma TAC and α-tocopherol and the proportion of n-3 PUFA in RBC indicates that n-3 PUFA are associated with higher antioxidant capacity in plasma and an increased concentration of one of the antioxidant vitamins.

The correlation found between plasma TAC and the proportion of n-3 PUFA in RBC may have depended on the wide range in the proportion of n-3 PUFA in RBC from the women participating in the study. No assessment was made of dietary intake in the present study. However, because the proportion of n-3 PUFA in RBC has been shown to reflect the dietary intake of n-3 PUFA in the preceding weeks or months (24), the high proportion of n-3 PUFA in RBC from many of the women probably reflects their intake of fish and/or fish oil. A high proportion of the women (37.4%) in the present study consumed fish oil supplements regularly, and one dietary survey (25) revealed that 36% of women in Iceland consumed fish at least three times a week in 2002.

Our results showing a positive correlation between plasma TAC and n-3 PUFA in RBC are in line with results showing that fish oil n-3 PUFA increase the antioxidant status in ulcerative colitis patients (3). However, our results are not in accordance with results from two intervention studies that show no effect of dietary fish oil supplements (18) or DHA supplementation (19) on plasma TAC, although in both studies supplementation resulted in increased plasma concentration of EPA and/or DHA. The discrepancy between the results from the present association study and the two intervention studies is not easily explained. One important factor that is different in all three studies is the consumption and/or plasma concentration of α-tocopherol. In the intervention studies participants consumed 206 mg/d (18) and 16.75 mg/d (19) of vitamin E, with intervention in the former study leading to an increased concentration of α-tocopherol in plasma but in the latter study to a trend toward reduced plasma α-tocopherol levels. In our study, women with a higher proportion of n-3 PUFA in the RBC had a higher concentration of α-tocopherol in the plasma. Thus, the differences in plasma concentrations of α-tocopherol do not explain the discrepancy between the results from the present association study and the two intervention studies.

The negative correlation seen between plasma TAC and the proportion of n-6 PUFA in RBC is probably explained by a strong negative correlation found between the proportions of n-3 and n-6 PUFA in RBC ($r = –0.643$, $P < 0.0001$). Although dietary n-3 PUFA supplementation is often thought to decrease the proportion of n-6 PUFA in phospholipids, this is not always the case (26,27), and when n-3 PUFA were added to the diet with n-6 PUFA, there was an increase in both n-3 and n-6 PUFA in the RBC in piglets (28). Our results, showing a strong negative correlation between n-3 and n-6 PUFA in RBC in a population with a mixed intake of n-3 and n-6 PUFA, indicate that in women with the highest proportion of n-3 PUFA in the RBC, there is enough n-3 PUFA in their diet to replace n-6 PUFA in the RBC.

The positive correlation between the plasma concentration of α-tocopherol and the proportion of n-3 PUFA in the RBC seen in the present study is in line with results from another

study showing an increased α-tocopherol concentration with the intake of fish oil supplements (18), which often have α-tocopherol added to them. The positive correlation between the plasma TAC and plasma concentration of α-tocopherol was expected, as α-tocopherol is known to contribute, although minimally, to the antioxidant capacity in the TEAC assay (21). The positive correlation between plasma TAC and the proportion of n-3 PUFA in the RBC is only partly explained by the positive correlation between plasma TAC and the concentration of α-tocopherol in plasma, as in a multivariate analysis with both the proportion of n-3 PUFA in RBC and α-tocopherol concentration in the plasma as independent variables, the proportion of n-3 PUFA in the RBC was an independent contributor to the correlation with plasma TAC.

The positive correlation between plasma TAC and age in the present study is in accordance with results from other studies (29,30). However, the positive correlations between plasma TAC and the plasma concentration of α-tocopherol and age are probably explained by the positive correlation between the proportion of n-3 PUFA in RBC and age (Fig. 2). The higher proportion of n-3 PUFA in the RBC of older birth cohorts may be due to a higher intake of fish oil supplements by older women than younger ones (data not shown). The higher proportion of n-3 PUFA in the RBC of older birth cohorts may also be due to greater fish consumption, as a dietary survey in Iceland in 2002 showed more fish consumption among older birth cohorts than younger ones (25). A trend toward a positive correlation between the proportion of n-3 PUFA in plasma phospholipids in Icelanders and age has previously been reported (31).

The positive association of plasma TAC with alcohol consumption in the present study is in line with results from intervention studies showing increased plasma TAC with wine consumption (32–36). However, the highest alcohol consumption in this study (two glasses of wine, beer, or spirits per day) was much lower than the consumption in an intervention study by van der Gaag *et al.* (37) (four glasses/d of red wine, beer, or spirits over a 3-wk period) that had no effect on TAC. About 20% of the participants did not respond to the question about alcohol consumption. Whether the women who did not respond to the question consumed more alcohol than the ones who did, and whether inclusion of their alcohol consumption would have affected the outcome of the study are not known. Also not known is whether the positive association between plasma TAC and alcohol consumption in the present study is linked to the intake of phenolic compounds, as has been suggested in other studies (34,38,39), as no distinction was made between beverages with or without phenolic compounds.

The results from the present study demonstrate a positive correlation between plasma TAC and the plasma concentration of α-tocopherol and the proportion of n-3 PUFA in RBC total lipids from 99 Icelandic women and a negative correlation between the plasma concentration of lycopene and the proportion of n-3 PUFA in RBC. A high proportion of n-3 PUFA in RBC reflects the dietary intake of n-3 PUFA from fish oil supplements and/or fish. Thus, the results suggest that dietary intake of n-3 PUFA may be accompanied by an increase in plasma TAC and does not lead to adverse effects on plasma concentrations of most of the antioxidant vitamins.

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Cholesterol-Lowering Ability of a Phytostanol Softgel Supplement in Adults with Mild to Moderate Hypercholesterolemia

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ABSTRACT: Plant sterols, incorporated into spreads and other food sources, have been shown to lower serum cholesterol concentrations. The effect of phytostanol supplementation in softgel form has not been assessed. Our objective was to examine the effects of sitostanol as sitostanol ester in softgel form on serum lipid concentrations in hypercholesterolemic individuals. Thirty hypercholesterolemic adults were supplemented with 1.6 g of free phytostanol equivalents as phytostanol ester (2.7 g stanol esters) or placebo per day for 28 d in a randomized, double-blind, parallel study design. Phytostanol supplementation resulted in a significant decrease in total cholesterol (TC) (–8%) and LDL-cholesterol (–9%). There were no alterations in concentrations of HDL-cholesterol or TG. Nor were the ratios of LDL/HDL or TC/HDL altered significantly. Thus, use of phytostanol ester softgel supplements improved serum total and LDL-cholesterol concentrations in hypercholesterolemic individuals.

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Dietary approaches to lowering heart disease risk factors are of interest to many in the population. The use of naturally occurring plant sterols is one such approach. Plant sterols are essential components of plant cell membranes with a structure similar to animal-derived cholesterol (1). Among the most abundant plant sterols are sitosterol, campesterol, and stigmasterol, which differ in their side chain configuration. Phytostanols are the completely saturated forms of phytosterols (2) and are not as abundant in nature. The Western diet contains approximately 100–300 mg sterols and 20–50 mg stanols per day (3), with the main sources being cooking oils, legumes, and seeds. Although cholesterol absorption from foods is as high as 20–80%, plant sterol absorption is likely less than 5% and stanol absorption less than 3% (3).

Plant sterol/stanol-enriched margarines are available on the market in many countries, and the ability of these products to lower circulating cholesterol concentrations is supported by multiple human feeding trials in normo- and hypercholesterolemic adults (3–7). Decreases in total and LDL-cholesterol concentrations of up to 15% have been observed.

Side effects of plant sterols/stanols appear to be rare and include reduced absorption of β-carotene, lycopene and α-tocopherol (8–10) as well as mild gastrointestinal complaints. Furthermore, the results of supplementation with plant sterol-/stanol ester-containing foods in combination with cholesterol-lowering medications, such as cerivastatin (11) and other statins (12), not only suggest a lack of increased side effects but also an increase in cholesterol-lowering ability equivalent to doubling the statin intake.

High-fat foods or spreads may not be the most appropriate form of phytostanol supplementation (13). Recently, the ability of phytosterols/stanols to reduce heart disease risk factors in non- or low-fat matrices has been investigated with differing results (14–18). Jones *et al.* (15) demonstrated that phytosterols added to nonfat milk or a low-fat beverage did not lower cholesterol levels to a greater extent than the control beverage. However, further research by this and other groups has demonstrated that phytosterols have total cholesterol (TC)- and LDL-lowering ability in a variety of media, including low-fat milk, yogurt, bread, cereal, and jam (14–18).

Although there is great potential for the phytosterol/stanol enrichment of a variety of foods, it is not always convenient for the consumer to incorporate a particular food into their daily diet because of cost, travel, or taste preferences. Thus, phytosterol/stanol softgel supplements have recently become available. These products are more convenient for most consumers. Although TC and LDL reduction is supported by studies investigating efficacy of food-based phytosterol/stanol-enriched products, there have been no published studies investigating lipid modification with phytosterol/stanol softgel supplements.

The purpose of this study was to examine whether supplementation with phytostanol ester softgel capsules for 28 d alters lipoprotein-cholesterol concentrations in men and women with elevated cholesterol. The hypothesis tested was that when a phytostanol ester dietary supplement is provided to moderately hypercholesterolemic adults for 4 wk, lipoprotein cholesterol profiles will be beneficially altered.

MATERIALS AND METHODS

Subjects. This study received ethical approval by the Research Ethics Board of the University of Guelph, Guelph, Ontario, Canada. Thirty adults (9 females, 21 males; aged 33–70 yr) with mild to moderate hypercholesterolemia (TC > 5.0 mmol/L

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Abbreviations: BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; TC, total cholesterol.

or 193 mg/dL) were selected for this study. Subjects were screened, after 12 h of fasting, for TC concentrations. Inclusion criteria included TC concentrations greater than 5.0 mmol/L (193 mg/dL), aged 18–70 yr, body mass index (BMI; kg/m^2) > 18 and < 34. Individuals were excluded if they were pregnant and/or lactating, were using hormone replacement therapy, had diabetes, were using cholesterol-lowering medication or had used cholesterol-lowering medication within the past 3 mon, had a past myocardial infarction or heart surgery, were allergic to the treatment, or had used street drugs within the past 3 mon. Subjects were randomly assigned, using a standard random number table, into the two treatment groups on the basis of serum cholesterol concentrations.

Protocol. This study was a randomized, double-blind, placebo-controlled clinical trial. All subjects were free living throughout the trial duration and were advised to maintain regular eating and exercise habits. At baseline, subjects reported to the laboratory after a 12-h overnight fast to receive orientation training, to have a baseline blood sample taken and for various anthropometric measurements such as height, weight, blood pressure (BP), and heart rate. Subjects were randomized, using a standard random number table, into 4 blocks based on TC levels—5.0–5.99 mmol/L (193–231 mg/dL), 6.0–6.99 mmol/L (232–270 mg/dL), 7.0–7.99 mmol/L (271–309 mg/dL), and 8.0 mmol/L (310 mg/dL) and greater—to receive either placebo or phytostanol supplements (BENECOL® SoftGels, McNeil Nutritionals, LLC, Fort Washington, PA). Although the capsules were not identical, the supplements were packaged in opaque bags by someone not involved with the study, to ensure that both the researchers and subjects remained blind. General feedback from subjects was that they did not know what treatment they were receiving, and the lack of availability of BENECOL® Softgel supplements in Canada made it difficult for subjects to have seen the capsules prior to the study.

Each group received instructions to consume 3 softgel (glycerin) capsules with breakfast and with dinner daily for 28 d. The placebo capsules contained 500 mg corn oil/capsule (0.75 mg phytostanol and 0.9 mg phytosterol per capsule). The label claim indicated that consumption of 6 phytostanol ester softgel capsules per day would result in supplementation of 2.0 g phytostanol as phytostanol esters per day. Results from an independent testing laboratory indicated that the phytostanol ester capsules contained 266 mg phytostanol equivalents and 5 mg phytosterol equivalents per capsule for a total of 1.6 g phytostanol equivalents (2.7 g stanol esters) and 30 mg phytosterol equivalents per day. GC analysis of the phytostanol ester softgel capsules indicated that they comprised 65% sitostanol, 34% campestanol, 1% sitosterol, and less than 1% campesterol.

Subjects received instructions on how to record all food eaten for 3 d on study days 1–3 and 26–28. During the study, one individual in the treatment group dropped out for reasons unrelated to the study. Subjects returned to the laboratory on day 29 after an overnight fast (12 h) for various anthropometric measurements and a blood sample. All food records were returned at this time. Subjects were provided with a phone number to contact study personnel throughout the study duration.

Analysis. Venous blood samples were collected, in 10 mL vacutainer tubes with no additive (Becton Dickinson Vacutainer System, Franklin Lake, NJ), from fasting subjects on days 0 and 29 of the trial. Serum was obtained after 15 min of centrifugation at 775 × *g* at 4°C (Universal 32R; Hettich Zentrifugen, Tuttlingen, Germany) and frozen at –20°C until analysis. All samples from each subject were sent to the Guelph General Hospital (Guelph, Ontario) and analyzed within the same analytic run. Serum TC and HDL-cholesterol, as well as serum TG concentrations, were determined quantitatively with a within-run percent variability of <3% using a Synchron CX9® system (Beckman Coulter, Fullerton, CA). TC and TG were determined with a Synchron CX Multi calibrator, the former at a ratio of 1 part sample to 100 parts reagent (Beckman Coulter 467825) and the latter at a ratio of 1 part sample to 100 parts reagent (Beckman Coulter 445850); for both the change in absorbance at 520 nm was determined. HDL-cholesterol was determined quantitatively with a Synchron systems CX Lipid calibrator at a ratio of 1 part sample to 93 parts reagent (Beckman Coulter 6520207); the change in absorbance at 520 nm was determined. The within-run percent variability was <3%. TG was quantitatively determined using a Synchron CX9® system with Synchron® systems CX Multi calibrator (Beckman Coulter). A ratio of 1 part sample to 100 parts reagent (Beckman Coulter 445850) was used. The change in absorbance at 520 nm was determined. The within-run percent variability was <3%. LDL-cholesterol was determined by the Friedewald equation (19). Conversion of mmol/L to mg/dL is based on multiplication of 38.67 for TC, LDL- and HDL-cholesterol and 88.57 for TG.

At each visit total body weight was assessed using a calibrated medical scale (Acculab SV-100, Edgewood, NY) accurate to 0.1 kg. In addition, height, BP (Oscillometric Blood Pressure monitor UA-767CN; Life Source, Milpitas, CA; accuracy of ±2%), and resting heart rate (Oscillometric Blood Pressure monitor UA-767CN; accuracy of ±5%) were taken at each visit. All pre- and post-experiment measurements were taken at approximately the same time of day by the same investigator. For BP and heart rate measurements, the cuff was placed approximately 2.5 cm above the elbow joint. All manufacturers' directions were followed. Subjects sat in a chair for 5 min prior to measurement. One reading was taken from the left arm. Approximately 30 s afterward, one measurement was taken from the right arm. The average of the two measurements was used.

Phytostanol and phytosterol levels in the supplement (BENECOL® and placebo) were determined as follows by Enviro-Test (Edmonton, Alberta, Canada). An internal standard of 2.5 mg/mL cholestanol (C-2882; Sigma, St. Louis, MO) in propanolol (HPLC grade) was prepared and stored at 4°C. A system suitability standard of 0.5 mg/mL cholestanol and 0.5 mg/mL stigmastanol (5–4297; Sigma) was prepared and stored at 4°C. The softgel capsules to be analyzed were sliced to expose centers and placed in a 250-mL volumetric flask. THF (200 mL) was added, and the mixture was sonicated in a 50°C ultrasonic bath for 90 min. The mixture was left to cool at room temperature. Then 1.0 mL was pipetted into a screw-cap test

tube and evaporated under nitrogen at room temperature. Next, 5.0 mL of internal standard, 15.0 mL of 95% ethanol, and 5.0 mL of concentrated KOH solution [60.09 KOH pellets (85%) in 40 mL distilled water] was added. The tubes were capped and samples were mixed vigorously using a vortex mixer for 60 s and then sonicated in a 70°C ultrasonic bath in a fume hood for 5 min. Tubes were removed from the bath, mixed vigorously using a vortex mixer for 60 s, and sonicated at 70°C for 60 min. After cooling to room temperature, tubes were mixed vigorously using a vortex mixer for 60 s to disperse the saponified solution uniformly and 2.0 mL was transferred to a screw-cap test tube. Distilled water (3.0 mL) and 6.0 mL hexane were added to each test tube containing saponified solution, and the solution was mixed vigorously using a vortex mixer for 60 s and centrifuged at 345 × *g* for 3 min. The top lipid layer was transferred to a silylation vial and was evaporated under nitrogen at 70°C. After cooling to room temperature, 200 μL silylation reagent [bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane, (99:1, w/w) and 100 μL pyridine were added. The tubes were left to silylate for 15 min at 70°C and cooled to room temperature. Samples were transferred to GC vials containing GC vial inserts for small volumes. Final GC sample preparations are stable in the GC vials for 72 h. GC (HP 5890 series II; Hewlett-Packard) column conditions were as follows: Column HP-5, 30 m long, 0.32 mm i.d., 0.25 μm film thickness; temperature program 300°C isothermal, 12 min; injector temperature 300°C; detector temperature 310°C, FID; helium 10–12 psi; make-up flow 20 mL/min; split ratio 1:50; injection volume 1 μL; syringe 10 μL. Stanol or sterol amount (mg/10 softgels) = [(area stanol)/(std amt) (df)]/area std, where area stanol = area of the stanol peak in the chromatogram, std amt = amount of the internal standard (mg), area std = area of the internal standard peak in the chromatogram, and df = dilution factor (200). Total stanols (mg) = amount sitostanol (mg) + amount campestanol (mg)

Statistical methods. Based on a power of 80% and a Type I error rate of $\alpha = 0.05$, a sample size of 13 participants per group was required to detect an estimated difference of 0.96 mmol/L. An approximate SD of 0.87 mmol/L has been observed in adults from previous work in this laboratory. That is,

$$n = \frac{(Z_\alpha + Z_\beta)^2 \times 2\sigma^2}{\Delta^2} \quad [1]$$

$$n = \frac{(1.96 + 0.84)^2 \times 2 \times 0.87^2}{0.96^2} = 13 \quad [2]$$

where Δ = change between subject's data at baseline and at day 28.

Data are expressed as means with SE. Assumptions for normality and homogeneity were tested and met with the exception of TG concentrations, which required log transformation. Comparison between groups was carried out with a two-sample *t*-test (20). Within-groups changes were analyzed using a two-sample paired *t*-test (20). *P* values less than 0.05 are considered significant.

RESULTS AND DISCUSSION

To our knowledge, this study is the first in which moderately hypercholesterolemic subjects, on self-selected diets, were supplemented with a softgel form of phytostanol esters. Previous studies have examined the effect of phytostanols/phytosterols in food sources such as margarine, or low-fat alternates such as bread, milk and other liquids (3–7,14–18).

Thirty subjects started the supplementation trial; 29 subjects completed the entire study. One study member dropped out for reasons unrelated to the study. Compliance was determined by capsule count at the end of the study. Average compliance for the treatment group was 95.5% and for the placebo group was 97.1%

All subjects were able to tolerate the phytostanol supplements with little or no adverse effects. Adverse effects reported, in the placebo group only, included a rash. Subjects reported that they were not aware of which supplementation group they were in despite the nonidentical appearance of the study capsules.

Three-day dietary analyses results are presented in Table 1. There were no differences in dietary consumption of total calories and cholesterol or percent protein, fat, carbohydrate, or alcohol between groups. In the control group, consumption of carbohydrate increased and alcohol decreased toward the end of the study (days 26–28) as compared with the beginning of the study (days 1–3).

TABLE 1
Dietary Analysis of the Phytostanol and Control Groups[a]

	Phytostanol		Control	
	Day 1–3	Day 26–28	Day 1–3	Day 26–28
Total calories (kcal)	2087 ± 392	2104 ± 556	2237 ± 345	2350 ± 521
Percent protein	16.3 ± 4.4	15.6 ± 3.3	15.4 ± 4.3	15.8 ± 2.2
Percent fat	30.3 ± 6.0	33.3 ± 5.4	34.4 ± 7.3	31.6 ± 6.4
Percent carbohydrate	49.3 ± 7.5	47.8 ± 6.1	45.5 ± 8.7	50.7 ± 7.1[b]
Percent alcohol	4.3 ± 4.5	3.3 ± 3.8	4.4 ± 4.7	2.0 ± 2.8[b]
Cholesterol (mg)	233 ± 103	247 ± 72	328 ± 162	276 ± 121

[a]Mean ± SD; *n* = 12 (phytostanol) and *n* = 15 (control); two subjects did not turn records in. There were no significant differences between groups at baseline.
[b]$P < 0.05$ vs. baseline.

TABLE 2
Effect of Phytostanol Supplementation on Anthropometric Measures in Moderately Hypercholesterolemic Adults[a]

	Phytostanol		Control	
	Day 0	Day 29	Day 0	Day 29
Weight (kg)	80.5 ± 10.6	80.6 ± 10.7	76.0 ± 9.3	75.6 ± 9.3[b]
Heart rate[c]	68.2 ± 8.7	68.2 ± 8.6	67.1 ± 10.1	67.5 ± 11.0
Systolic pressure	136 ± 16.6	138 ± 16.4	139 ± 16.6	139 ± 17.2
Diastolic pressure	86 ± 8.1	86 ± 12.1	86 ± 7.9	85 ± 6.9

[a]Mean ± SD; $n = 14$ (phytostanol) and $n = 15$ (control).
[b]$P < 0.05$ vs. baseline.
[c]Beats per minute.

Baseline measurements for all subjects are given in Tables 2 and 3. Ages ranged from 33 to 70 yr and were balanced for gender (10 males in each group). The average age for the study was 53.7 (±10.1) yr. The average BMI was 27.5 ± 3.4 kg/m^2. BP ranged from 106 to 167 mmHg (systolic) and 76–105 mmHg (diastolic) and TC levels from 5.0 to 8.2 mmol/L (193–317 mg/dL). There were no differences in baseline values between the groups and subjects were randomized based on screening TC values (data not shown).

There were no differences in weight, resting heart rate, or BP at baseline between the two groups. Furthermore, there was no effect of phytostanol supplementation on weight, resting heart rate, or BP (Table 2). There was a slight, but significant decrease in weight in the control group over the 28-d study period.

Concentration of TC, HDL, LDL and TG did not differ between control and phytostanol-supplemented study groups at baseline (Table 3). Phytostanol supplementation resulted in a significant decrease in TC (–8%) and LDL-cholesterol (–9%). There were no alterations in concentrations of HDL-cholesterol or TG. Nor were the ratios of LDL/HDL or TC/HDL altered significantly. Thus, supplementation with approximately 1.6 g phytostanols in ester form (2.7 g stanol esters), as softgels, was effective in lowering circulating TC and LDL-cholesterol concentrations. The reduction of TC and LDL-cholesterol by phytostanols in this form is in agreement with results of other investigators in which phytostanols/phytosterols in high- and low-fat food forms were effective in reducing cholesterol concentrations (3–7,14–18).

The efficacy of phytostanols/phytosterols in lowering TC and LDL-cholesterol concentrations has been shown for both phytostanol and phytosterol, either esterified or nonesterified (reviewed in Refs. 3,13). Most studies have investigated the efficacy of these products in margarine form (4–7), as the use of a high-fat carrier was thought to be advantageous. Recently, the desire for lower-fat food carriers and/or alternative food choices has resulted in the investigation of the ability of phytosterols to lower cholesterol concentrations in food products such as bread, milk, juice, yogurt, and tortilla chips, among others (14–18,21). The addition of properly solubilized nonesterified plant sterols to low-fat milk was shown to result in decreased cholesterol absorption in mildly hypercholesterolemic men (14). Clifton *et al.* (16) showed that consumption of 1.6 g phytosterols per day as phytosterol esters, as part of many low-fat products, including milk, yogurt, bread, and cereal, all resulted in lowered cholesterol concentration, although the low-fat milk was the most effective (15% reduction vs. 5–9% for other products). Decreased LDL-cholesterol concentrations were observed in additional nonmargarine studies involving sterol ester fortification of bakery products (22), tortilla chips deep-fried in phytosterol-enriched oil (21), and chocolate (23). Not all studies involving nonmargarine food sources of supplemented phytosterol are supportive of cholesterol concentration lowering, however. Jones *et al.* (15) compared the cholesterol-lowering ability of a nonfat beverage vs. beverages with additional phytosterols. There was no additional cholesterol lowering as a result of phytosterol supplementation as compared with nonfat beverage consumption.

TABLE 3
Effect of Phytostanol Supplementation on Cardiovascular Disease Risk Factors in Moderately Hypercholesterolemic Adults[a]

	Phytostanol		Control	
	Day 0	Day 29	Day 0	Day 29
Total cholesterol (mmol/L)[b]	6.92 ± 1.0	6.39 ± 1.0[c]	6.87 ± 0.8	6.72 ± 0.6
LDL-cholesterol (mmol/L)	5.35 ± 1.0	4.86 ± 1.0[c]	5.21 ± 0.7	5.11 ± 0.6
HDL-cholesterol (mmol/L)	1.22 ± 0.1	1.17 ± 0.2	1.26 ± 0.3	1.22 ± 0.3
TG (mmol/L)	1.79 ± 0.7	1.80 ± 1.0	2.02 ± 1.5	1.93 ± 1.1
LDL/HDL	4.51 ± 1.2	4.28 ± 1.1	4.35 ± 1.1	4.42 ± 1.2
TC/HDL	5.82 ± 1.3	5.63 ± 1.3	5.72 ± 1.3	5.77 ± 1.4

[a]Mean ± SD; $n = 14$ (phytostanol) and $n = 15$ (control).
[b]Conversion to mg/dL × 38.67 for total, LDL- and HDL-cholesterol and × 88.57 for TG.
[c]$P < 0.05$ vs. baseline.

Previous research demonstrated that Cytellin®, a nonesterified sitostanol drug in capsule form marketed by Eli Lilly, which was available in the United States from the 1950s to 1980s, significantly decreased TC and LDL-cholesterol at a recommended dose of 6–12 g per day (1). The present study showed that phytostanol ester in softgel form at a dose of 1.6 g per day is effective at lowering TC and LDL-cholesterol concentrations. After 28 d, TC concentrations were decreased by 8% and LDL-cholesterol concentrations by 9%. This translates into a 9–18% lower risk of cardiovascular disease (CVD) based on a 1–2% CVD risk reduction for each 1% reduction in LDL-cholesterol (24). This degree of reduction was comparable to reductions in LDL-cholesterol noted after consumption of sterol-enriched milk and yogurt for 3 wk (16), as well as 4-wk consumption of sterol-enriched chocolate (23), and is more than after consumption of sterol-enriched bread and cereal (16).

The label claim indicated that consumption of 6 softgel capsules per day resulted in supplementation of 2.0 g phytostanol as phytostanol esters (3.4 g stanol esters) per day. Results from an independent testing laboratory indicated that in reality a daily intake of 1.6 g per day of phytostanol as phytostanol esters was achieved. Other studies demonstrating TC and LDL-cholesterol concentration-lowering efficacy of phytostanols or phytosterols have used between 0.8 and 3 g per day (13). Thus, the doses used in this study, at either the label-indicated dose or the independent test dose, were well within levels previously shown to decrease serum TC and LDL-cholesterol values.

A recent meta-analysis of 18 clinical trials showed that consumption of plant sterols/stanols incorporated into spreads led to decreased LDL-cholesterol concentrations of 8–13% (10). Furthermore, it has been suggested that nonesterified phytostanols may provide better cholesterol-lowering potential than nonesterified phytosterols (4,6,25,26). This is perhaps owing to the decreased intestinal absorption of the hydrogenated phytostanols. Esterification, however, may result in similar cholesterol-lowering abilities of the sterols and stanols (7,27,28). It is likely that esterification of the stanol/sterol leads to higher incorporation into mixed micelles, thereby more effectively inhibiting dietary and bile cholesterol absorption as compared with nonesterified stanol or sterol powders.

The mechanism by which plant sterols/stanols exert their hypocholesterolemic action is not clearly understood. Plant sterols/stanols have been shown to decrease cholesterol absorption from the intestine (29–31). Cholesterol absorption may be inhibited by as much as one-third with phytostanol/sterol supplementation (30). Although the inhibition of cholesterol absorption may increase internal cholesterol synthesis, resulting decreased serum cholesterol concentrations suggest that the increased synthesis does not compensate for decreased absorption. The individuals in this study were told to consume the stanol ester-containing softgels with meals to take this into consideration. Although not specifically determined, it can be hypothesized that the phytostanol esters in the supplement inhibited cholesterol absorption from the accompanying meal and bile contents in the intestine. Furthermore, this study was not designed to determine whether results would differ if the capsules were taken on an empty stomach. Future studies could be designed to answer these and other questions, including optimal dosing (for example, a single daily dose vs. divided dosages).

In summary, the results of the present study show the efficacy of a softgel form of phytostanol esters in lowering TC and LDL-cholesterol in the absence of negative effects on HDL-cholesterol and fasting TG concentrations. Furthermore, these changes were noted in the absence of dietary modification and were in the same order of magnitude as those reported in various studies in which individuals consumed phytostanol- and/or phytosterol-enriched nonmargarine food products for 3–4 wk. We conclude that the use of phytostanol ester softgel supplements by mildly to moderately hypercholesterolemic subjects may be useful in sole or adjunct LDL-cholesterol-lowering therapy, thus reducing CVD risk.

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Dose-Dependent Hypocholesterolemic Actions of Dietary Apple Polyphenol in Rats Fed Cholesterol

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ABSTRACT: The dose-dependent hypocholesterolemic and antiatherogenic effects of dietary apple polyphenol (AP) from unripe apple, which contains approximately 85% catechin oligomers (procyanidins), were examined in male Sprague-Dawley rats (4 wk of age) given a purified diet containing 0.5% cholesterol. Dietary AP at 0.5 and 1.0% levels significantly decreased the liver cholesterol level compared with that in the control (AP-free diet-fed) group. Dietary AP also significantly lowered the serum cholesterol level compared with that in the control group. However, the HDL cholesterol level was significantly higher in the 1.0% AP-fed group than in the control group. Accordingly, the ratio of HDL-cholesterol/total cholesterol was significantly higher in the 0.5% AP-fed group and 1.0% AP-fed group than in the control group. Moreover, the atherogenic indices in the 0.5 and 1.0% AP-fed groups were significantly lower than those in the control group. The activity of hepatic cholesterol 7α-hydroxylase tended to be increased by dietary AP in a dose-dependent manner. In accord with this observation, dietary AP increased the excretion of acidic steroids in feces. Dietary AP also significantly promoted the fecal excretion of neutral steroids in a dose-dependent manner. These observations suggest that dietary AP at a 0.5 or 1.0% level exerts hypocholesterolemic and antiatherogenic effects through the promotion of cholesterol catabolism and inhibition of intestinal absorption of cholesterol.

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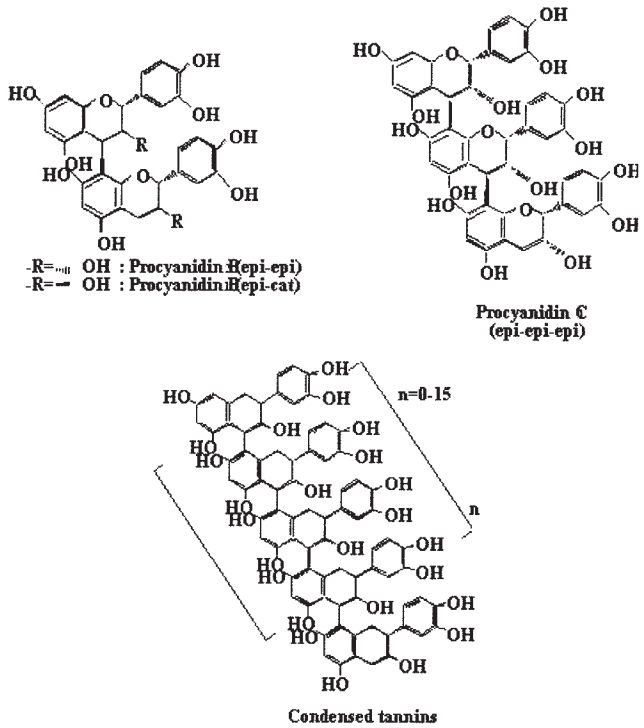
Many polyphenolic compounds such as catechin and its derivatives, oligomeric catechins (procyanidins), may have beneficial effects on human health and preventive effects against diseases such as life-style-related diseases, without causing severe side effects.

A high level of plasma cholesterol has been ranked as one of the greater risk factors contributing to the incidence and severity of coronary heart disease. Previous studies showed that tea catechin exhibited hypocholesterolemic (1) and hypolipidemic effects (2) in experimental animals. Moreover, a combination of niacin-bound chromium and natural grape seed procyanidins, having 54% dimeric, 13% trimeric, and 7% tetrameric oligomeric procyanidins, also decreased total cholesterol and LDL levels in hypercholesterolemic subjects (3). Thus, dietary procyanidins, which are catechin oligomers, and catechin may prevent cardiovascular heart disease through hypocholesterolemic effects.

Humans consume approximately 28 mg of polyphenols/d from various plant foods and beverages (4). The total polyphenol level in a typical fruit serving of 200 g is 50–500 mg (5), and apples contain over 200 mg per serving of polyphenols. Thus, apples can serve as a major source of dietary polyphenols. Boyer and Liu (6) summarized various health benefits of apple phytochemicals; however, that review only showed functions of ripe apple and monomeric polyphenols. Unripe apples contain significantly higher levels of procyanidins than ripe apples (7); however, these procyanidins have not been used in foods yet. The procyanidins in unripe apple are 2–14mer oligomeric (−)-epicatechins, and they constitute over 60% of the total polyphenols in apples (7). Scheme 1 shows structures of procyanidins in apple polyphenol (AP) from unripe apple. The regulative effects of dietary procyanidins on lipid metabolism, especially on cholesterol metabolism, have not been completely evaluated yet. However, our recent study showed that



SCHEME 1

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Abbreviations: AP, apple polyphenol; CYP7, cholesterol 7α-hydroxylase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.

TABLE 1
Composition of Dietary Apple Polyphenol (AP)

Component	Degree of polymerization	Apple polyphenol (wt%)
Procyanidins		
Procyanidin	2mer	11.1
Procyanidin	3mer	12.3
Procyanidin	4mer	8.7
Procyanidin	5mer	5.9
Procyanidin	6mer	4.9
Procyanidin	>7mer	20.9
Procyanidin	>8mer	18.9
Sum catechin oligomers		82.7
Other flavonoids (monomeric polyphenols)		
(–)-Epicatechin		6.1
(+)-Catechin		1.1
Phloridzin		1.1
Phloridzin xyloglucoside		2.7
Chlorogenic acid		4.8
p-Coumaroylquinic acid		1.5
Sum		17.3

dietary procyanidins exerted hypolipidemic and antiobesity effects in experimental animals in which obesity was induced by consumption of high levels of lard (8). Therefore, the consumption of high doses of procyanidins may contribute to improvement of the lipid profile in hypercholesterolemic subjects. Here we examined the dose-dependent hypocholesterolemic effect of dietary purified AP [containing oligomeric (–)-epicatechin] from unripe apple in rats fed cholesterol to evaluate the preventive effects against atherogenesis and cardiovascular heart disease. We also examined the dose-dependent antioxidative effect of dietary AP.

EXPERIMENTAL PROCEDURES

Chemicals. The composition of dietary AP is shown in Table 1. AP contains approximately 85% procyanidins. This material was purified and provided by Asahi Brewery Co., Ltd. (Tokyo, Japan). [4-^{14}C]Cholesterol (52 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Radioactive cholesterol was purified by TLC before use.

Animals and diets. Male Sprague-Dawley strain rats (3 wk old, CLEA Japan, Co., Ltd. Tokyo, Japan) were housed individually in a room with controlled temperature (20–23°C) and light (0800–2000). After rats were acclimatized for 1 wk, they were divided into four groups consisting of eight or nine rats each and given the following diets containing 0.5% cholesterol, as shown in Table 2: polyphenol-free diet (control); 0.2% AP-supplemented diet (AP-0.2); 0.5% AP-supplemented diet (AP-0.5); 1.0% AP-supplemented diet (AP-1.0). Each diet was prepared according to the AIN93 recommendations (9). The group fed the diet without polyphenol was given the diet at the level consumed by the AP-1.0 group under pair-feeding conditions because dietary polyphenol may cause a slight reduction in food intake. After 30 d, rats were anesthetized lightly using diethylether and bled from the abdominal aorta, and various tissues were quickly excised. Plasma was prepared by centrifugation after allowing blood to clot at room temperature. The liver was excised immediately and microsomes were prepared at 4°C. Ten days before the rats were killed, feces were collected for 2 d from each rat and then lyophilized and subsequently analyzed for the levels of neutral and acidic steroids in feces.

The samples were stored at −80°C until analyses. The animal experiments were conducted according to the guidelines of the ethical committee for experimental animal care at Hirosaki University.

Measurement of hepatic cholesterol 7α-hydroxylase (CYP7) activity. The activity of hepatic CYP7 was measured by the modified method of Van Cantfort *et al.* (10). The level of microsomal protein was measured by the method of Bradford (11).

Lipid analyses. Liver and serum lipids were extracted by the method of Folch *et al.* (12), and the levels of phospholipids, cholesterol, and TG in liver and serum were measured as described previously (13). The levels of serum TG and HDL-cho-

TABLE 2
Diet Composition

	Group			
Component[a]	C (wt%)	AP-0.2 (wt%)	AP-0.5 (wt%)	AP-1.0 (wt%)
Cornstarch	41.25	41.05	40.75	40.25
Casein	20	20	20	20
α-Cornstarch	13.2	13.2	13.2	13.2
Sucrose	10	10	10	10
Soybean oil	5	5	5	5
Cellulose	5	5	5	5
Mineral mix[b] (AIN93)	3.5	3.5	3.5	3.5
Vitamin mix[b] (AIN93)	1	1	1	1
L-Cystine	0.3	0.3	0.3	0.3
Choline bitartrate	0.25	0.25	0.25	0.25
Cholesterol	0.5	0.5	0.5	0.5
AP	—	0.2	0.5	1

[a]For abbreviation see Table 1.
[b]From Reference 9.

lesterol were measured using TG-E and HDL-cholesterol-E tests (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), respectively. HDL-cholesterol-E test is commercial kit based on the theory of Burstein *et al.* (14) and is applied to various animal experiments.

Analysis of steroids excreted into feces. The levels of fecal neutral and acidic steroids were analyzed by GLC using 5α-cholestane (neutral steroids analysis) and 23-nordeoxycholic acid (acidic steroids analysis) (Steraloids, Wilton, NH) as internal standards according to the modified method of Sugano *et al.* (15). Briefly, fecal steroids were extracted by hot ethanol and separated into neutral or acidic steroids after deconjugation in an autoclave using 1.25 M NaOH. Neutral steroids were extracted by diethyl ether, and then acidic steroids were extracted by diethyl ether after acidification by additon of HCl. Neutral steroids were analyzed on a Shimadzu GC-14B gas chromatograph with an FID using an EC-1 column (0.25 mm × 30 m, film thickness 0.25 μm; Alltech Inc., Deerfield, IL) after trimethylsilylation. The oven and injector temperatures were 270 and 300°C, respectively. Acidic steroids were analyzed on the same column with an FID after methylation by diazomethane and followed by acetylation by acetic anhydride. The oven and injector temperatures were 280 and 320°C, respectively.

Analysis of tissue lipoperoxide and α-tocopherol levels. Liver and serum lipoperoxides were measured by the method of Fukunaga *et al.* (16) as TBARS. The levels of serum and liver α-tocopherol were measured by the method of Ueda and Igarashi (17).

Northern hybridization analyses. Total RNA was isolated from fresh liver (100–200 mg) using the RNeasy Midi Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). mRNAs encoding CYP7 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, used as an invariant control) were analyzed by Northern hybridization with the respective oligonucleotide probes described below. Preparation and hybridization of ^{32}P-labeled DNA probes were carried out as described by Sambrook and Russel (18). The rat cDNA probes for CYP7 and GAPDH were prepared by ^{32}P-labeling a 300-bp fragment (DDBJ/Genebank accession number J05430) amplified using CYP7 oligonucleotide primers (upstream primer, 5′-GAGTGATGTTTGAAGCAACTGGC-3′; downstream primer, 5′-GTTCGCTTCTTCCAACCACG-3′) and a 700-bp fragment (DDBJ/Genebank accession number AB017801) amplified using GAPDH oligonucleotide primers (upstream primer, 5′-GCCATCAACGACCCCTTCATT-3′; downstream primer, 5′-CGCCTGCTTCACCACCTTCTT-3′), respectively.

RNA was separated by electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde and 20 mM MOPS. After electrophoresis, RNA was blotted onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom).

Quantification of the band intensities. The hybridization signals were visualized and the band intensity was quantified using a Bio-Imaging Analyzer BAS 1000 (Fuji Photo Film, Tokyo, Japan). Quantified mRNA levels of CYP7 were divided by those of GAPDH for normalization.

Statistical analyses. All values are presented as mean ± SE. Statistical comparisons between groups were analyzed by Duncan's new multiple-range test (19) and Super ANOVA software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as $P < 0.05$.

RESULTS

Growth and liver weight. On average, a rat weighing 96 g was fed 16.5 g of purified diet per day and gained 147–158 g of body weight during 30 d (Table 3). The weight gain in the AP-0.2 group was significantly higher than that in the control group; however, no such increase was seen in the AP-0.5 and AP-1.0 groups. The average liver weight in the AP-0.5 group was significantly lower than that in the control group.

Lipid levels in liver and serum. The level of liver TG tended to be lowered by dietary AP in a dose-dependent manner compared with that in the control group, although the decrease was not significant (Fig. 1). Dietary AP at the levels of 0.5 and 1.0% significantly lowered the liver cholesterol level compared with that in the control group. There was no significant difference in the liver phospholipid level among the groups.

Dietary AP tended to decrease the serum TG level, although the effect was not significant (Fig. 2). The level of serum phospholipid was highest in the AP-1.0 group among all of the groups. Dietary AP significantly decreased the level of serum cholesterol compared with that in the control group. The level of HDL-cholesterol in the AP-1.0 group was significantly higher than that in the control group, although HDL-cholesterol was lower in the AP-0.2 group than in the control group. The ratio of HDL-cholesterol/total cholesterol was significantly higher in the AP-0.5 and AP-1.0 groups than in the control group. Moreover, the atherogenic indices (the ratio of non-HDL

TABLE 3
Growth Parameter[a]

Group	Initial body weight (g)	Weight gain (g)	Food intake (g/30 d)	Liver weight (g/100 g B.W.)
Control	96 ± 2	147 ± 3[a]	496 ± 3	3.5 ± 0.1[a]
AP-0.2	96 ± 2	158 ± 3[b]	497 ± 3	3.3 ± 0.1[a,b]
AP-0.5	96 ± 2	148 ± 3[a,b]	497 ± 5	3.2 ± 0.1[b]
AP-1.0	96 ± 2	153 ±4[a,b]	497 ± 7	3.3 ± 0.1[a,b]

[a]Data are presented as mean ± SE of 8 or 9 rats in each group. Values without a common superscript letter are significantly different at $P < 0.05$ (Duncan's new multiple-range test). B.W., body weight; for other abbreviation see Table 1.

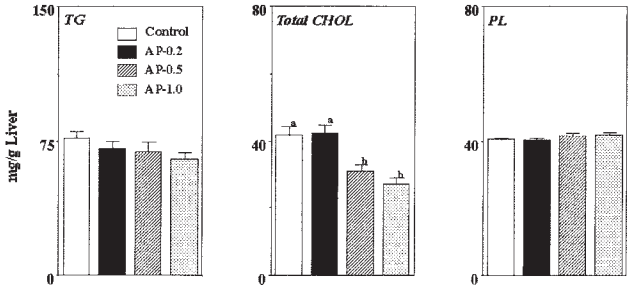

FIG. 1. Dose-dependent effect of dietary AP on liver lipid levels. Each value represents the mean ± SE for data obtained from 8 or 9 animals. Values without a common superscript letter are significantly different at $P < 0.05$. Abbreviations: AP, apple polyphenol; Control, rats fed AP-free diet; AP-0.2, rats fed 0.2% AP-supplemented diet; AP-0.5, rats fed 0.5% AP-supplemented diet; AP-1.0, rats fed 1.0% AP-supplemented diet; total CHOL, total cholesterol; PL, phospholipids.

cholesterol/HDL cholesterol) in the AP-0.5 and AP-1.0 groups, but not in the AP-0.2 group, were significantly lower than that in the control group.

TBARS and α-tocopherol levels in serum and liver. The level of TBARS in the liver tended to be lowered in a dose-dependent manner by consumption of AP compared with the level in the control group (control, 49.33 ± 3.63 nmol/g; AP-0.2, 46.88 ± 4.28 nmol/g; AP-0.5, 43.35 ± 1.91 nmol/g; and AP-1.0, 42.14 ± 1.34 nmol/g). Dietary AP at the 0.2 and 0.5% levels also significantly lowered the serum TBARS level compared with that in the control group (control, 1.29 ± 0.07 nmol/mL; AP-0.2, 1.09 ± 0.02 nmol/mL; AP-0.5, 1.09 ± 0.05 nmol/mL; and AP-1.0, 1.18 ± 0.03 nmol/mL). On the other hand, dietary AP increased the level of α-tocopherol in a dose-dependent manner (control, 1.93 ± 0.13 μg/mL; AP-0.2, 2.14 ± 0.16 μg/mL; AP-0.5, 2.16 ± 0.15 μg/mL; and AP-1.0, 3.09 ± 0.19 μg/mL). Such an effect did not necessarily occur in the liver, although dietary AP at the 0.2% level significantly increased the level of liver α-tocopherol compared with that in the control group (control, 25.83 ± 1.11 μg/g; AP-0.2, 28.78 ± 1.62 μg/g; AP-0.5, 27.57 ± 2.06 μg/g; and AP-1.0, 25.85 ± 1.38 μg/g).

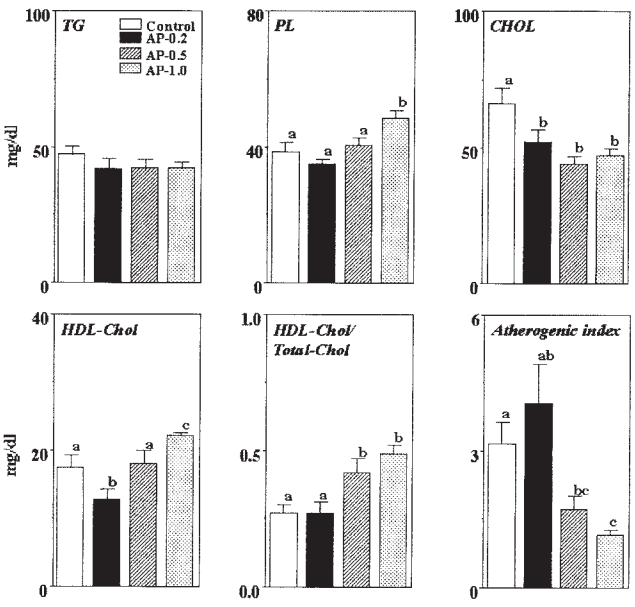

FIG. 2. Dose-dependent effect of dietary AP on serum lipid levels. Each value represents the mean ± SE for data obtained from 8 or 9 animals. Values without a common superscript letter are significantly different at $P < 0.05$. HDL-cholesterol-E test, which is a commercial kit based on the theory of Burstein *et al.* (14), was used for HDL-cholesterol analysis. For abbreviations see Figure 1.

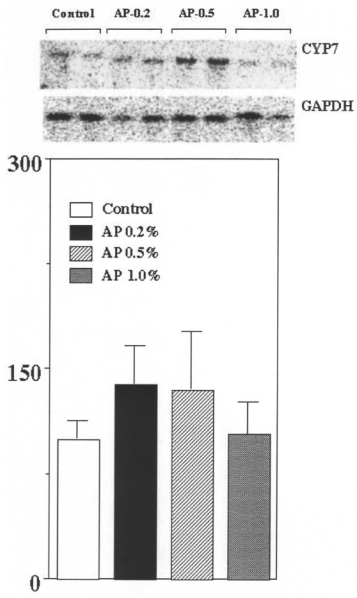

FIG. 3. Dose-dependent effect of dietary AP on hepatic cholesterol 7α-hydroxylase (CYP7) mRNA levels in rats fed 0.5% cholesterol for 30 d. Each value represents the mean ± SE for data obtained from 8 or 9 animals. The value of the CYP7 mRNA signal was normalized to the value of the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) signal, and each value is expressed relative to the average value for the rats fed the control diet, which was set to 1. For other abbreviations see Figure 1.

Hepatic CYP7 activity. The hepatic CYP7 activity was significantly higher in the AP-0.5 and AP-1.0 groups than in the control group (control, 15.7 ± 2.7 pmol/min/mg protein; AP-0.2, 19.2 ± 2.6 pmol/min/mg protein; AP-0.5, 23.8 ± 1.6 pmol/min/mg protein; and AP-1.0, 22.4 ± 2.0 pmol/min/mg protein)

Level of CYP7 mRNA. The level of CYP7 mRNA in the liver tended to be higher in the AP-0.2 and AP-0.5 groups than in the control group; however, the increases were not significant and no increase was seen in the AP-1.0 group (Fig. 3).

Fecal steroid excretion. The levels of steroids excreted into the feces are summarized in Figure 4. The level of neutral steroids excreted into the feces was increased by consumption of AP in a dose-dependent manner. Dietary AP, especially in the AP-0.5 and AP-1.0 groups, also increased the excretion of acidic steroids into feces compared with that in the control group.

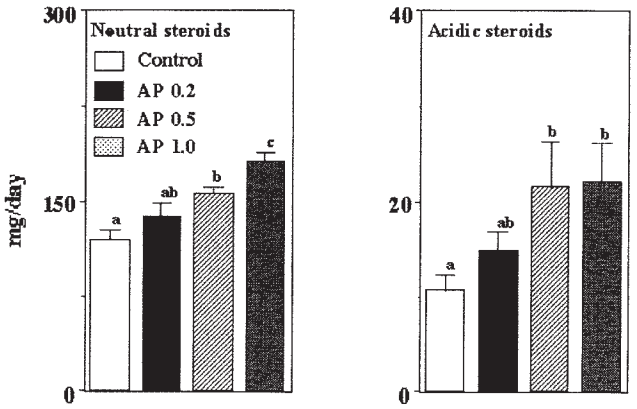

FIG. 4. Dose-dependent effect of dietary AP on neutral and acidic steroid excretion into feces. Each value represents the mean ± SE for data obtained from 8 or 9 animals. Values without a common superscript letter are significantly different at $P < 0.05$. For abbreviations see Figure 1.

DISCUSSION

Many reports show various health benefits of dietary apple or beverages from apple because it has phytochemicals such as polyphenol or pectin (6). Owing to the many studies on the biological activities of apple, the antioxidative effects of AP are now well known (20,21). On the other hand, Leontowicz *et al.* (22,23) and Aprikian *et al.* (24,25) report detailed and interesting data concerning the regulative effects of apple on lipid profiles. However, direct evidence of the regulative activities on lipid metabolism by AP has not been completely shown until now because these studies used juice or extracts from ripe apple. Moreover, the biological activity of procyanidins, which is high in apple, also has not yet been completely evaluated.

Therefore, we examined the dose-dependent hypocholesterolemic and antioxidative effects of dietary purified AP from unripe apple, which contained 84.9% (−)-epicatechin oligomers (procyanidins), in rats fed 0.5% cholesterol in this study. Our dose level of AP to rats is between 0.2 and 1.0%, and these levels are high; however, the elucidation of dietary functions of polyphenols such as tea catechin containing a high level of (−)-epigallocatechin gallate were carried out with the dose levels of 0.5, 1.0, or 2.0% in recent studies (26). No toxic effects were reported in these studies. Therefore, we also evaluated the effects of a high dose of AP on lipid metabolism in rats in this study.

Dietary AP increased weight gain at the 0.2% level and lowered liver weight at the 0.5% level; however, no impairment of hepatic functions (glutamic-oxaloacetic transaminase, glutamic pyruvic transaminase, and lactate dehydrogenase, data not shown) was observed at the 0.5 or 1.0% AP level, and thus a high dose of (−)-epicatechin oligomers such as that in AP appears not to be toxic. Shoji *et al.* (27) also reported that an acute oral-toxicity test, and a 90-d subchronic-toxicity test showed no significant hematological, clinical, chemical, histopathological, or urinary effects at a dose of 2 g/kg. Therefore, a high dose of AP, at 1.0% may exert no side effects or toxic effects.

Antioxidative activity of dietary AP was observed in these animal experiments, as already shown in many *in vitro* studies (6, 20, 21). Similar observations were made in human studies using grape seed extracts (28) or cocoa powder (29). The antioxidative action of dietary AP was significant in serum when the rats were given AP at the 0.2 or 0.5% level. This effect may be related to the free radical-scavenging action of metabolites from dimers and trimers of AP, which are soluble polyphenols in water and alcohol, incorporated into chylomicrons although their bioavailabilities are not necessarily high. Effects of monomeric polyphenols such as (−)-epicatechin and chlorogenic acid as shown in Table 2 also cannot be ignored; however, they are present at very low levels compared with the procyanidins in the AP that we used. Moreover, Lotito and Frei (30) showed that monomeric polyphenols in ripe apple and extracts from ripe apple did not exert antioxidative effects *in vivo* as shown in *in vitro* studies. Contrary to this report, Facino *et al.* (31) observed that diets supplemented with procyanidins from *Vitis vinifera* (grapes) enhanced plasma antioxidative activity in rats. Therefore, the plasma antioxidative activity in rats fed AP should be evaluated.

The level of serum α-tocopherol was higher in AP-fed groups than in that in control group. Dietary (+)-catechin and BHT markedly increased the level of α-tocopherol in rats by a mechanism independent of tocopherol-ω-hydroxylase, which is a key enzyme in tocopherol catabolism (32). Therefore, the metabolites of procyanidins, rather than internal α-tocopherol, may play critical free radical-scavenging roles. However, α-tocopherol level in liver was decreased in a dose-dependent manner, although their levels were higher than that in control group. These observations were not shown in serum α-tocopherol level. We must carry out a supplementary examination to elucidate this contradiction.

Dietary AP at levels of 0.5 and 1.0% significantly lowered the level of cholesterol in the liver compared with that in the control group. These effects may be related to the modulation of hepatic CYP7 activity by consumption of AP. The effects of dietary polyphenol on hepatic cholesterol catabolism have not been completely elucidated yet. However, the consumption of Lung Chen tea, which is a type of Chinese green tea, tends to lower the hepatic CYP7 and liver cholesterol levels in rats fed 1% cholesterol (33). Moreover, the consumption of a 4% Lung Chen tea solution significantly increased the excretion of fecal acidic steroids compared with that in the control (no consumption of tea) group. Sable *et al.* (34) also observed that fecal excretion of bile acids and neutral steroids increased in hamsters fed apple. Similar to this observation, the fecal excretion of acidic steroids was significantly higher in the AP-0.5 and AP-1.0 groups than in the control group in our study, reflecting the modulation of hepatic CYP7 activity. The polyphenolic compounds in Lung Chen tea are catechin monomers such as (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, and (−)-epigallocatechin gallate. Therefore, the metabolites of procyanidins from AP, which are oligomers of (−)-epicatechin, as well as catechin monomer, may modulate hepatic CYP7 activity and consequently increase the excretion of acidic steroids

into feces. This effect of dietary procyanidins must contribute to the decrease of the cholesterol level in the liver. The level of CYP7 mRNA was not necessarily associated with the hepatic CYP7 activity, although the consumption of 0.2 and 0.5% AP tended to increase the level of CYP7 mRNA. Therefore, dietary AP may modulate the expression of CYP7 in liver; additional evidence will be needed to determine the precise relationships among dietary AP, cholesterol catabolism, and the expression of CYP7 mRNA, because the level of CYP7 mRNA was not consistent with the activity of hepatic CYP7 in this study.

Dietary AP may exert a hypocholesterolemic effect through promoting the excretion of neutral steroids into the feces. Tea catechins exert a hypocholesterolemic effect through the inhibition of lymphatic cholesterol absorption in rats (35). This observation was attributed to the reduction of cholesterol solubility in mixed micelles by tea catechins. Moreover, an earlier study also showed that dietary crude tea catechin powder decreased plasma total cholesterol, cholesterol ester, VLDL-cholesterol + LDL-cholesterol, and atherogenic index through the increase of fecal excretion of total lipids and cholesterol in rats fed lard and cholesterol (36). Thus, dietary procyanidins from apple may exert hypocholesterolemic effects through the inhibition of intestinal absorption *via* the reduction of the micellar solubility of cholesterol.

These results suggest that it is possible that dietary purified procyanidins from unripe apple exert cholesterol-lowering effects in liver and serum through the combination of the activation of hepatic cholesterol catabolism and the inhibition of intestinal cholesterol absorption.

Solvents such as water, alcohol, and acetone can extract low- and intermediate-MW polyphenolic compounds such as hydrolyzable tannins and procyanidins (37). However, high-MW compounds remain insoluble in the same solvents (37). In their review, Bagchi and Preuss (38) estimate that monomeric polyphenol, and oligomeric procyanidin fractions, which are soluble in water and alcohol, may be partially absorbed from the intestine and detected in all tissues and plasma. On the other hand, high-MW procyanidin fractions, which are insoluble in water and alcohol but soluble in aqueous acetone, may be excreted in the feces and not detected in the plasma or tissues (38). Therefore, the soluble fractions in AP may affect hepatic cholesterol catabolism, whereas the insoluble fractions in AP may promote cholesterol excretion by inhibiting intestinal absorption.

The metabolic pathway of procyanidins has not been completely elucidated. For example, Baba *et al.* (39) observed that procyanidins B2 were absorbed and excreted in urine, and a portion of the procyanidins B2 is degraded to (−)-epicatechin and to the metabolized conjugated and/or methylated (−)-epicatechin internally in the rat. Sano *et al.* (40) also observed that procyanidins B1 were detected in human serum 2 h after intake. Similarly, the absorption of orally administered procyanidins B2 and B3 from Cinnamomi cortex in rat plasma was reported (41). On the other hand, Tsang *et al.* (42) reported that the metabolites from (−)-epicatechin and low amounts of the procyanidin dimers (B1, B2, B3, and B4) as well as the trimer (C2) and an unknown trimer in rats given grape seed extract; however, they indirectly estimate that procyanidins are not depolymerized to monomers after consumption. Moreover, some reports estimate that the metabolites such as phenolic acids from procyanidins metabolized by intestinal microflora contribute to various biological activities of dietary procyanidins because many phenolic acids were excreted into urine (43,44). Therefore, further experiments are needed to elucidate why dietary AP exerts hypocholesterolemic and antioxidative effects.

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Effects of Margarine and Butter Consumption on Distribution of *trans*-18:1 Fatty Acid Isomers and Conjugated Linoleic Acid in Major Serum Lipid Classes in Lactating Women

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ABSTRACT: *Trans* FA (TFA) have at least one *trans* double bond and comprise several isomers and types, including many of the CLA (e.g., *c*9,*t*11-18:2 CLA). Some TFA may have adverse effects (e.g., cardiovascular disease), whereas some are thought to have beneficial effects (e.g., anticarcinogenicity). The presence of TFA in human tissues and fluids is related to dietary intake, although this relationship is not completely understood—especially in regard to serum lipid fractions. This study was conducted as part of an investigation designed to test the influence of butter (B), "low TFA" margarine (LT), and regular margarine (RM) on milk fat content. Here we tested the secondary hypothesis that consumption of B, LT, and RM by lactating women would result in differential distribution of TFA and CLA in major serum lipid classes. Breastfeeding women (n = 11) participated in this randomized Latin-square study consisting of five periods: intervention I (5 d), washout I (7 d), intervention II (5 d), washout II (7 d), and intervention III (5 d). Extracted serum lipid was separated into cholesterol ester (CE), TAG, and phospholipid (PL) fractions and analyzed for total and isomeric TFA and CLA concentrations. Data indicate that TAG consistently contained the highest concentration of total *t*-18:1. No interaction between treatment and fraction was found for any of the *t*-18:1 isomers identified. Absolute concentration of each *t*-18:1 isomer was greatest during the RM period, regardless of fraction. On a relative basis, concentrations of *t*10-18:1 and *t*12-18:1 were most responsive to treatment in the CE fraction. The concentration of *c*9,*t*11-18:2 CLA was highest in the TAG fraction and lowest in the PL fraction, regardless of treatment. In summary, these results indicate (i) that there is a differential distribution of some isomeric TFA and CLA among human serum lipid fractions and (ii) that dietary TFA intake influences absolute and relative concentrations of some of the isomers in selected fractions.

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Trans FA (TFA) are present in a variety of foods; some are derived from natural sources such as dairy products (1), but most come from products that contain commercially hydrogenated oils (2). The most prevalent TFA in partially hydrogenated vegetable oils (PHVO) tends to be *t*10-18:1, and the major TFA isomer in dairy products is *t*11-18:1 (3,4). Metabolic studies show that some TFA have adverse effects on coronary heart disease (CHD) risk factors, increasing LDL cholesterol while decreasing HDL cholesterol (5–8). A positive correlation also has been found between TFA intake and cholesterol level and CHD death rate (5,9–11). In addition to influencing risk of CHD, some TFA may have adverse effects on fetal and infant growth and development. For example, Rosenthal and Doloresco (12) demonstrated that TFA impaired the microsomal desaturation and chain elongation of the EFA to their long-chain polyunsaturated metabolites. These long-chain FA with 20 and 22 carbon atoms, such as arachidonic acid and DHA, are of great physiological importance during perinatal development. Some TFA have also been shown to depress milk fat concentration when administered to dairy cows or humans (13,14). Estimates of TFA intake among Americans vary, but data from Kris-Etherton and colleagues (15) suggest that TFA intakes range from 2.6 to 12.8 g/d. Allison *et al.* (16) reported that the U.S. population consumes 5.3 g/d TFA, representing 2.6% of total energy intake and 7.4% of energy from fat. In view of the potential deleterious effects of some TFA, the Institute of Medicine recently recommended that the TFA intake be a low as possible (17).

It is noteworthy that CLA were excluded from this recommendation, although many of CLA isomers are TFA. In general, CLA refers to a group of PUFA that exist as positional and stereoisomers of conjugated octadecadienoic acid (18:2). The predominant CLA isomer in foods is *c*9,*t*11-CLA, also called rumenic acid (18), and is found primarily in ruminant-derived foods such as beef, lamb, and dairy products (19–21). Data suggest that CLA intake in the U.S. population averages approximately 151 and 212 mg/d in females and males, respectively, with the majority (68%) coming from dairy products (22). Synthetic mixtures of CLA also can be readily purchased as nutritional supplements and are composed primarily of the *c*9,*t*11- and *t*10,*c*12-18:2 isomers. Unlike most other TFA, numerous beneficial health effects have been attributed to CLA, including those related to its potential antiadipogenic, antidiabetogenic, anticarcinogenic, and antiatherosclerotic properties (23–26). For this reason, CLA was not considered by the Institute of Medicine to be a TFA that should be avoided.

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Abbreviations: B, butter; CE, cholesterol ester; CHD, coronary heart disease; LT, "low TFA" margarine; MTBE, methyl tert-butyl ether; PHVO, partially hydrogenated vegetable oil; PL, phospholipid; RM, regular margarine; TFA, *trans* FA.

The FA composition of the diet has been shown to influence the FA composition of serum lipid fractions such as TAG, cholesterol ester (CE), and phospholipid (PL) as well as that of adipose tissue (27,28). However, very few studies have reported the individual TFA isomers in plasma, let alone in each lipid fraction (29,30). We recently reported (31) a combination of methods that could be used to methylate the CLA content of various plasma lipid fractions. However, we did not identify any of the *t*-18:1 isomers in the plasma lipid fractions. Doing this is important, because it is possible that the distribution of various TFA among the lipid fractions might have implications on human health. The influence on health of the incorporation of TFA might be different according to whether TFA are incorporated preferentially into the CE or the PL fraction. Thus, the overall objective of this study was to determine the effect of diet on the distribution of isomeric *t*-18:1 and CLA in the major plasma lipid fractions of humans. This study was part of a larger investigation designed to test the effect of the consumption of butter (B), low-TFA margarine (LT), and regular margarine (RM) on milk fat content. Results of this study have been published elsewhere (14). Thus, we were able to test the hypothesis that consumption of these commonly consumed fats by lactating women would alter the distribution of TFA isomers and CLA in major human serum lipid classes. We also tested the hypothesis that consumption of RM would increase the concentration of most of the *t*-18:1 isomers in each lipid class.

MATERIALS AND METHODS

Materials. Heneicosanoate (21:0), a mixture of 19 FAME, a mixture of CLA isomers (FFA; 80% *c*9,*t*11-18:2, 17% *c*9,*c*11-18:2, and 1% *t*9,*t*11–18:2) and a *t*-18:1 mixture (*t*6-, *t*7-, *t*9-, *t*11-, *t*12-, and *t*13-18:1) were purchased from Matreya (Pleasant Gap, PA). Sodium methoxide in methanol (0.5 N) and methyl acetate were obtained from Sigma-Aldrich Co. (St. Louis, MO). Silica Maxi-Clean™ cartridges (600 mg) were purchased from Alltech Associates, Inc. (Deerfield, IL). All chemicals and reagents used were of analytical grade.

Subjects. All procedures were approved by the Washington State University Institutional Review Board, and all participants provided written consent for themselves and their infants. Healthy, lactating women (n = 12) from 2 to 12 mon postpartum were recruited from the Pullman, WA, and Moscow, ID, areas. However, serum samples from only 11 subjects were analyzed in this study, as there was insufficient serum from one subject. Baseline data pertaining to these subjects are presented in Table 1. All women were self-reported to be healthy and were, on average, 26.7 ± 1.2 yr of age.

Study design and dietary interventions. This study was designed as a randomized Latin-square study consisting of five periods: intervention I (5 d), washout I (7 d), intervention II (5 d), washout II (7 d), and intervention III (5 d). Initial data analyses (not shown) suggested that there were no carryover effects from one intervention period to the next; thus, only data from the intervention periods (not washout periods) are presented here.

TABLE 1
Demographic Variables and Anthropometric Measurements of Women and Infants[a]

Variables	Mean ± SEM
Maternal	
Age (yr)	26.7 ± 1.2
Time postpartum (mon)	6.1 ± 1.0
Weight (kg)	70.5 ± 4.3
Height (cm)	167.7 ± 1.7
BMI (kg/m^2)	24.9 ± 1.2
Body fat[b] (%)	31.3 ± 2.0
Infant	
Weight (kg)	7.9 ± 0.5
Length (cm)	66.9 ± 1.7

[a]Anthropometric measurements were taken at time of enrollment; n = 11.
[b]Body fat was measured by dual energy X-ray absorptiometry. BMI, body-mass index.

During each intervention period, subjects consumed diets high in either B, RM, or LT. During the B period, subjects maximized their consumption of B and minimized their intake of PHVO. During the RM period, subjects maximized their consumption of RM and minimized their intake of dairy fats. During the LT period, subjects maximized their intake of LT and limited their intake of both dairy fats and PHVO. To facilitate compliance with these requests, subjects were provided with 454 g of either butter (Darigold® Sweet Cream Butter, Grade AA; Darigold, Inc., Seattle, WA), regular margarine (Gregg's™ Gold-n-Soft® margarine; Ventura Foods, City of Industry, CA), or margarine labeled as containing no TFA (Saffola Soft Margarine®;Ventura Foods) during the corresponding intervention period.

In addition, subjects consumed one "breakfast" muffin and one "lunch" muffin daily, each made with 37.8 ± 1.0 g of the appropriate fat. Four different types of muffins were prepared and systematically rotated for variety and enhanced subject compliance. Breakfast and lunch muffins were consumed before 1000 and 1600 h, respectively, except on day 5 of each intervention period, when subjects ate the breakfast muffin 1 h before the blood collection time, and the lunch muffin before 1200 h. Data concerning the nutrient and FA intake from the study muffins are summarized in Table 2. Total fat intakes were 123.1, 120.8, and 125.6 g/d during B, LT, and RM periods, respectively. No dietary restriction was imposed during the washout periods, and subjects were allowed to eat according to their choices.

Blood collection. Nonfasting maternal blood (20 mL) was collected by venipuncture on the last day of each intervention between 0800 and 1000 h. Blood was drawn approximately 1 h after consumption of the breakfast muffin. Serum samples were aliquoted and frozen for later analyses.

Lipid extraction, fractionation, and transmethylation. Lipids were extracted according to the method of Ingalls et al. (32) as modified by us (31). Extracted lipid (1.25 mg) was fractionated in quadruplicate using silica cartridges and combined after fractionation. Briefly, the column was loaded with 1.5 mg of plasma lipid; CE and TAG fractions were eluted with a combination of hexane/methyl tert-butyl ether (MTBE) (200:3, 12

TABLE 2
Total Nutrient and FA Provided by Muffins Consumed Daily During Each Intervention Period

Dietary component	Dietary fat source used to produce muffins		
	Butter (B)	"Low TFA" margarine (LT)	Regular margarine (RM)
Energy (kcal)[a]	1130	1129	1150
Protein (g)[a]	9	9	9
Carbohydrate (g)[a]	136	135	135
Lipid (g)[a]	62	61	64
Saturated FA (g)[b]	38	12	13
Cholesterol (mg)[a]	195	35	35
Sodium (mg)[a]	1492	1465	1599
10:0 (mg)[b]	1335	21	ND
12:0 (mg)[b]	1475	1475	5
14:0 (mg)[b]	4905	235	55
15:0 (mg)[b]	450	10	ND
16:0 (mg)[b]	16060	4130	7350
*c*9-16:1 (mg)[b]	565	80	55
18:0 (mg)[b]	8430	5220	5220
*c*9-18:1 (mg)[b]	10245	25745	7815
*t*6/*t*8-18:1 (mg)[b]	350	857	2171
*t*9-18:1 (mg)[b]	418	1149	2576
*t*10-18:1 (mg)[b]	728	1210	3464
*t*11-18:1 (mg)[b]	1431	1219	4017
*t*12-18:1 (mg)[b]	630	610	2285
*t*13/14-18:1 (mg)[b]	506	378	1198
*t*16-18:1 (mg)[b]	555	178	507
*c*9,*c*12-18:2 (mg)[b]	3310	10125	19350
*c*9,*t*11-18:2 CLA (mg)[b]	257	80	224
*t*10,*c*12-18:2 CLA (mg)[b]	24	33	75

[a]Values estimated from information provided on food labels.
[b]Values derived from biochemical analyses. ND, not detectable.

mL for CE followed by 12 mL for TAG). After removal of TAG, the column was acidified with 12 mL hexane/acetic acid (100:0.2). Finally, the PL fraction was eluted with 20 mL MTBE/methanol/ammonium acetate (pH 8.6; 10:4:1, by vol). TAG and PL fractions were dried under nitrogen at 45°C and transmethylated using sodium methoxide and methyl acetate for 10 min at room temperature; CE fractions were transmethylated similarly for 1 h (33).

Analysis of FAME. FAME were analyzed on a gas chromatograph (Hewlett-Packard 6890 Series) with auto injector fitted with an FID (Agilent Technologies, Wilmington, DE). FA profiles were determined by split injection (20:1) onto an SP-2560 fused-silica capillary column (100 m × 0.25 mm i.d., 0.2 μm film thickness; Supelco, Inc. Bellefonte, PA) using a programmed temperature gradient method (34). The helium carrier gas pressure was constant, and the injector and detector temperatures were 255°C. The initial oven temperature was 70°C and was held for 4 min. Following injection of the sample, the oven temperature was increased at 4°C/min to 170°C and held for 3 min. The oven temperature was then increased at 1°C/min to 185°C and held for 20 min and then increased at 3°C/min to 215°C and then 10°C/min to 230°C and held for 10 min, after which it was returned to 70°C. Identities of total *t*-18:1 isomers (sum of *trans* 6/8, *t*9, *t*10, *t*11, and *t*12 isomers), *c*9,*t*11-18:2 CLA, and other FA were established by comparing retention times to a 19 FAME standard mixture, and a CLA mixture containing 80% *c*9, *t*11-18:2; 17% *c*9,*c*11-18:2, and 1% *t*9,*t*11-18:2, respectively. Heneicosanoate (21:0) was added as an internal standard after serum fractionation to quantify the FAME concentration. We could identify only one CLA isomer that corresponded to the *c*9,*t*11-18:2 standard; *t*10,*c*12-18:2 was below the detection limit of our method. Similarly, only the major *t*-18:1 isomers (*trans* 6/8, *t*9, *t*10, *t*11, and *t*12 isomers) were identified in this study.

Statistical analyses. Standard descriptive statistics and ANOVA were performed using the Statistical Analysis System (Version 6.2; SAS, Cary, NC). The model used to test the stated hypotheses included the following factors: treatment, lipid fraction, and treatment × lipid fraction. Main effects and differences between means were considered significant at $P < 0.05$. Interactions were considered significant at $P < 0.05$.

RESULTS

Effects of treatment and lipid fraction on saturated and cis-FA. Effects of treatment on FA concentrations of lipid fractions are presented in Table 3. There was an interaction between treatment and fraction for the following FA: 14:0, 15:0, 16:0, 16:1, 17:0, and *c*9,*c*12,*c*15-18:3, whereas 12:0, *c*9-18:1, and *c*9,*c*12-18:2 had significant independent effects of both fraction and treatment ($P < 0.05$). Myristoleic acid (14:1), 18:0, and *c*5,*c*8,*c*11,*c*14-20:4 had a significant effect of fraction only ($P < 0.05$). During all periods, the predominant FA in CE was *c*9,*c*12-18:2 followed by *c*9-18:1. Oleic acid (*c*9-18:1) was the

TABLE 3
Effect of Dietary Treatment on FA Composition (mg/g lipid) of Human Plasma Lipid Fractions in Lactating Women[a]

	Fraction and treatment									
	CE[b]			TAG			PL[b]			Pooled
FA	B[b]	LT[b]	RM[b]	B	LT	RM	B	LT	RM	SEM
12:0[c,d]	0.2[a,b]	0.2[a,b]	0.1[a]	0.4[c]	0.3[b]	0.2[a,b]	0.0[a]	0.0[a]	0.0[a]	0.1
14:0[e]	1.5[c,d]	0.9[a,b,c,d]	0.8[a,b,c,d]	5.0[e]	1.7[d]	1.3[b,c,d]	0.5[a,b]	0.4[a]	0.8[a,b,c]	0.3
*c*9-14:1[d]	0.4[b,c]	0.2[a,b,c]	0.4[b,c]	0.4[c]	0.1[a,b,c]	0.1[a,b,c]	0.1[a,b]	0.0[a]	0.0[a]	0.1
15:0[e]	0.5[b]	0.3[a]	0.3[a,b]	0.8[c]	0.3[a,b]	0.3[a]	0.3[a]	0.2[a]	0.3[a]	0.0
16:0[e]	19.9[a,b]	14.3[a]	21.8[a,b]	31.9[c]	21.6[a,b]	19.1[a,b]	21.8[a,b]	24.9[b,c]	27.5[b,c]	3.2
*c*9-16:1[e]	3.6[d,e]	2.6[c]	2.1[c]	3.7[e]	2.7[c,d]	2.0[b,c]	0.5[a]	0.6[a]	1.1[a,b]	0.3
17:0[e]	0.3[a,b,c]	0.2[a]	0.3[a,b,c]	0.6[d]	0.3[a,b,c]	0.3[a,b]	0.4[b,c]	0.4[b,c]	0.5[c]	0.1
18:0[d]	4.9[a,b]	2.5[a]	7.9[b]	8.2[b,c]	6.4[a,b]	5.3[a,b]	12.6[c,d]	15.3[d]	14.4[d]	1.6
*c*9-18:1[c,d]	24.9[c,d]	28.5[d,e]	21.9[b,c,d]	36.9[e]	52.0[f]	28.8[d,e]	8.3[a]	12.5[a,b]	16.3[a,b,c]	4.1
*t*6/*t*8-18:1[c,d]	0.2[a,b]	0.1[a]	0.6[b,c]	0.6[a,b,c]	0.8[c]	1.6[d]	0.2[a,b]	0.3[a,b]	0.6[b,c]	0.1
*t*9-18:1[c,d]	0.2[a]	0.1[a]	0.4[a,b]	0.5[a,b]	0.9[b,c]	1.3[d]	0.2[a]	0.3[a,b]	0.7[b,c]	0.1
*t*10-18:1[c,d]	0.1[a,b]	0.1[a]	0.6[b,c,d]	0.9[d,e]	1.2[e]	1.9[f]	0.1[a,b]	0.3[a,b,c]	0.8[c,d,e]	0.2
*t*11-18:1[c,d]	0.2[a]	0.2[a]	0.6[a,b,c]	0.8[b,c,d]	1.1[d,e]	0.5[a,b,c]	0.4[a,b]	1.3[e]	0.9[c,d,e]	0.2
*t*12-18:1[c,d]	0.2[a,b]	0.1[a]	0.7[d,e]	0.6[b,c,d]	0.5[b,c,d]	1.2[f]	0.3[a,b,c]	0.6[c,d]	1.0[e,f]	0.1
*c*9,*c*12-18:2[c,d]	66.5[c]	64.6[c]	67.0[c]	14.4[a]	19.7[a,b]	29.4[b]	16.7[a,b]	22.4[a,b]	27.9[b]	4.6
*c*9,*t*11-18:2 CLA[d]	0.3[b,c]	0.2[a,b]	0.3[a,b,c]	0.7[d]	0.4[b,c]	0.5[c]	0.1[a]	0.1[a]	0.3[a,b,c]	0.1
*c*9,*c*12,*c*15-18:3[e]	0.7[a,b,c]	0.9[b,c]	0.9[b,c]	1.0[c]	2.5[d]	1.9[d]	0.21[a]	0.4[a,b]	0.7[a,b,c]	0.2
*c*5,*c*8,*c*11,*c*14-20:4[d]	7.4[a]	6.1[b,c]	7.3[c]	0.8[a]	0.7[a]	1.6[a]	4.7[b]	6.0[b,c]	6.0[b,c]	0.9
Total *t*-18:1[c,d]	0.9[a]	0.7[a]	2.9[b,c,d]	3.3[b]	4.4[d]	7.3[e]	1.1[a,b]	2.1[a,b,c]	4.0[c,d]	0.7

[a]Values represent means ($n = 11$). Means within treatments not sharing a common superscript are different ($P < 0.05$).
[b]CE, cholesterol ester; PL, phospholipids; B, butter; LT, "low *trans*" margarine; RM, regular margarine.
[c]Treatment effect ($P < 0.05$).
[d]Fraction effect ($P < 0.05$).
[e]Treatment × fraction interaction ($P < 0.05$).

predominant FA in the TAG fraction, except during the RM period when *c*9,*c*12-18:2 was instead. Palmitic acid (16:0) was the principal FA in the PL fraction, except during the RM period when it was *c*9,*t*12-18:2. Thus, although there were some relatively consistent trends as to which FA was the major one in each fraction, this was somewhat dependent on fraction and dietary treatment.

Effects of treatment and lipid fraction on total t-18:1 and t-18:1 isomers. The effects of treatment and lipid fraction on TFA are also presented in Table 3. There was no interaction between treatment and fraction on total *t*-18:1 concentration (defined as the sum of *t*6/8-, *t*9-, *t*10-, *t*11-, and *t*12-18:1). The TAG fraction contained the highest concentration of total *t*-18:1 followed by PL and CE (Fig. 1). There was also an independent effect of treatment ($P < 0.05$) in that consumption of RM resulted in higher total *t*-18:1 concentrations compared with the B and LT treatment periods.

We examined the effects of treatment and fraction on TFA isomers in two ways: (i) as absolute values (mg/g lipid) (Table 3) and (ii) as relative values (percentage of total *t*-18:1) (Table 4). When absolute values were considered, we found no interaction between treatment and fraction for any of the *t*-18:1 isomers identified. However, significant independent effects ($P < 0.05$) of fraction and treatment were evident for each isomer. In general, the concentration of each *t*-18:1 isomer was highest in the TAG fraction and lowest in the CE fraction and tended to be greatest during the RM period, regardless of fraction.

When each TFA was considered as a percentage of total *t*18:1, it was clear that the *t*10-18:1 isomer was the predominant TFA in the TAG fraction, regardless of dietary treatment. However, *t*11-18:1 was the second-most abundant during the B and RM period, whereas *t*9-18:1 was second-most abundant during the LT period. For the PL fraction, *t*12-18:1 was the most abundant during the LT and RM periods, with *t*11-18:2 being prominent during B consumption. The TFA profile of the CE fraction appeared to be most influenced by dietary treatment: *t*6/*t*8-18:1, *t*11-18:1, and *t*12-18:1 were the predominant TFA during B, LT, and RM periods, respectively.

Effects of treatment and lipid fraction on c9,t11-18:2 CLA. There was no interaction between treatment and fraction on

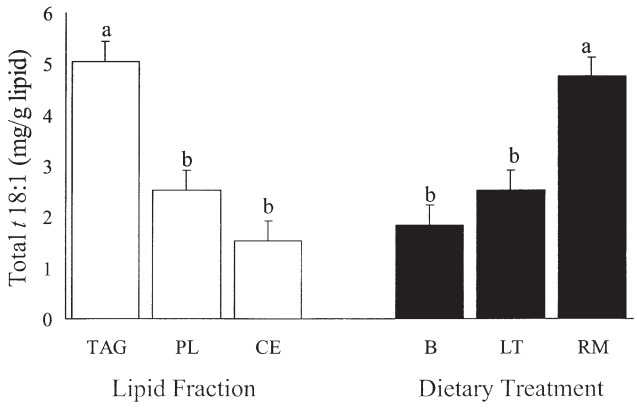

FIG. 1. Distribution of total serum *t*18:1 FA among lipid fractions [TAG; phospholipid (PL); and cholesterol ester (CE)] and by treatment (butter, B; "low TFA" margarine, LT; and regular margarine, RM). Each bar represents a mean ± SEM. Means within the lipid fraction or treatment groups not sharing a common letter differ significantly ($P < 0.01$).

TABLE 4
Distribution of *t*-18:1 Isomers of Plasma Lipid Fractions in Lactating Women[a]

	Lipid fraction[b]									
	CE			TAG			PL			Pooled
FA (% of total *t*-18:1)	B	LT	RM	B	LT	RM	B	LT	RM	SEM
*t*6/*t*8-18:1[c,d]	24.5[b]	18.6[a,b]	18.7[a,b]	17.3[a]	18.8[a,b]	19.8[a,b]	14.8[a]	15.1[a]	15.5[a]	2.2
*t*9-18:1[c,d]	21.9[b]	19.2[a,b]	14.2[a]	14.0[a]	22.9[b]	18.0[a,b]	13.7[a]	16.5[a,b]	16.7[a,b]	2.4
*t*10-18:1[c,d]	9.2[a]	16.5[a,b]	20.7[b,c]	26.6[b,c]	26.7[b,c]	25.3[b,c]	13.2[a]	14.7[a,b]	18.9[b,c]	2.8
*t*11-18:1[c,d]	23.4[a]	25.3[a,b]	19.2[a]	24.3[a,b]	19.6[a]	20.3[a]	32.1[b]	24.8[a,b]	23.3[a]	2.8
*t*12-18:1[c,d]	21.0[b,c,d]	20.4[b,c]	27.3[e]	17.8[a,b]	12.0[a]	16.6[a,b]	26.2[c,d,e]	28.8[e]	26.0[c,d,e]	2.4

[a]Values represent means ($n = 11$). Means within a lipid fraction not sharing a common superscript are different ($P < 0.05$).
[b]For abbreviations see Table 3.
[c]Treatment effect ($P < 0.05$).
[d]Fraction effect ($P < 0.05$).

*c*9,*t*11-18:2 CLA, nor was there an overall treatment effect. Nonetheless, consumption of B significantly increased the *c*9,*t*11-18:2 CLA content of the TAG fraction without changing the CLA content of the other fractions. There was a significant effect of fraction on *c*9,*t*11-18:2 CLA ($P < 0.05$), such that its concentration was highest ($P < 0.01$) in TAG (0.55 ± 0.04 mg/g lipid) followed by CE (0.31 ± 0.04 mg/g lipid) and PL (0.18 ± 0.44 mg/g lipid).

DISCUSSION

This study was conducted as a part of a larger investigation designed to test the influence of B, LT, and RM on milk fat in humans. In the present study we analyzed the distribution of *t*-18:1 isomers and *c*9,*t*11-18:2 CLA in major serum lipid fractions. Data indicate that the *t*-18:1 isomers and *c*9,*t*11-18:2 CLA were not equally distributed in TAG, PL, and CE fractions of serum lipid. The overall concentration of individual *t*-18:1 isomers was highest in the TAG fraction followed by PL and CE in each intervention period, respectively. Thus, it appears that the incorporation of *t*-18:1 isomers into serum lipid fractions is somewhat selective. Furthermore, overall concentration of total *t*-18:1 was also highest in the CE, TAG, and PL fractions during RM compared with the B and LT periods.

The results presented here for total TFA distribution among serum lipid fractions are in agreement with those of Vidgren *et al.* (35), who also reported a higher proportion of total TFA in the TAG fraction followed by PL and CE. Similarly, Schrock and Connor (36) reported the highest incorporation of total TFA into the TAG fraction followed by PL and CE in rabbits. However, in these studies isomeric TFA distributions were not reported. Recently, Mansour *et al.* (29) conducted a crossover intervention trial in which free-living subjects ($n = 10$) consuming moderate fat (25.6 ± 2.2% of energy from margarine) and then very low fat (9.9 ± 1.4% of energy from lean beef) diets were studied. The PL fraction contained more than 60% of the *trans*-18:1 isomers in plasma lipid in all subjects. The authors suggested that the measurement of *trans*18:1 in the plasma PL fraction could be used to estimate the presence or absence of TFA intake since it contained a greater amount of TFA compared with other fractions. Our results show that the FA concentration in CE tended to be most responsive to alterations in dietary TFA intake. Further, data suggest that incorporation of TFA isomers into individual lipid fractions is somewhat selective. Emken *et al.* (37) have also reported a selective discrimination in the incorporation of the *t*11-18:1 isomer compared with the *t*9-18:1 isomer in the TAG, CE, and FFA fractions.

We also studied the incorporation of CLA into lipid fractions. Recently, we reported (31) that the TAG fraction as opposed to the fractions CE and PL contained the highest percentage of CLA in human plasma. Thus, the present study confirms these findings and also supports those of others (38,39). We found that the effect of *c*9,*t*11-18:2 CLA intake on the amount of *c*9,*t*11-18:2 CLA in the lipid fractions was variable, with the TAG fraction appearing to be most responsive. However, this study was unable to take into account the endogenous conversion of *t*11-18:1 to *c*9,*t*11-18:2 CLA, which has been shown to occur (40). Clearly, stable isotope studies will be required to understand the partitioning of these FA, their precursors, and their metabolites in the body. Animal investigations also indicate that CLA accumulates predominantly in neutral lipids (41,42). In contrast, results from a recent study in Greek adults suggest that the majority of CLA is present in the PL fraction (43). Although most of the human studies indicate that CLA accumulates in the TAG, the discrepancy among study results remains unresolved.

In summary, these findings support our hypotheses that (i) concentrations of isomeric TFA and CLA in human serum lipid fractions are not equally distributed and (ii) maternal consumption of RM increases the level of *t*-18:1 isomers in the major lipid fractions. Interestingly, concentrations of CLA were less influenced by diet, although the conversion of *t*11-18:1 to *c*9,*t*11-18:2 CLA could not be accounted for here. Further studies are required to understand the biological significance of these findings.

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Effect of Substitution of Low Linolenic Acid Soybean Oil for Hydrogenated Soybean Oil on Fatty Acid Intake

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ABSTRACT: Low linolenic acid soybean oil (LLSO) has been developed as a substitute for hydrogenated soybean oil to reduce intake of *trans* FA while improving stability and functionality in processed foods. We assessed the dietary impact of substitution of LLSO for hydrogenated soybean oil (HSBO) used in several food categories. All substitutions were done using an assumption of 100% market penetration. The impact of this substitution on the intake of five FA and *trans* FA was assessed. Substitution of LLSO for current versions of HSBO resulted in a 45% decrease in intake of *trans* FA. Impacts on other FA intakes were within the realm of typical dietary intakes. No decrease in intake of α-linolenic acid was associated with the use of LLSO in place of HSBO because LLSO substitutes for HSBO that are already low in α-linolenic acid.

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As of January 2006, food manufacturers in the United States are required to comply with a new Food and Drug Administration regulation to label products with their *trans* FA content in the Nutrition Facts panel (1). This new regulation responds to the recognition among scientists that *trans* FA have unfavorable effects on blood cholesterol levels and thus increase the risk for coronary heart disease (2). The 2005 Dietary Guidelines for Americans recommend that consumers keep their intakes of *trans* FA as low as possible (3). Food manufacturers have already begun to respond by reformulating foods that contain hydrogenated vegetable oils, the main source of *trans* FA in the diet, to contain other oils with lower or no *trans* FA.

Using traditional breeding methods, developers have created a new variety of soybean that produces oil with a reduced level of α-linolenic acid (18:3n-3). Such soybean oil requires less or no hydrogenation to be used in many processed foods, thus providing opportunities for consumers to reduce their intakes of *trans* FA. It is possible that using this new soybean oil to reformulate foods could result in changes in the intake of other FA. The purpose of this study was to estimate the impact of replacing hydrogenated soybean oil (HSBO) in several current food applications with low linolenic acid soybean oil (LLSO) or a hydrogenated version of low linolenic soybean oil (H-LLSO) on the intakes of total *trans* FA and of five FA.

The key difference in composition of oil between low linolenic acid soybeans and traditional soybeans is the α-linolenic acid content: about 2.5% vs. about 7% of FA. Changes in other FA are small and within the range of variability of traditional soybean oil. However, because traditional soybean oil must be hydrogenated to be used in many food applications whereas LLSO can be used without hydrogenation, the FA content of foods made with LLSO will be different, most notably with respect to *trans* FA, compared with foods made with HSBO.

MATERIALS AND METHODS

Food consumption and composition data sources. Detailed food consumption data for the U.S. population were obtained from the USDA's Continuing Survey of Food Intakes by Individuals (CSFII), 1994–1996, and 1998 (4). Each person reported food intakes for two nonconsecutive days. For these analyses, an average daily intake over the 2 d was computed for each of five FA: palmitic, stearic, oleic, linoleic (all-*cis* isomer), and α-linolenic (all-*cis* isomer), and for total *trans* FA (the sum of *trans* isomers of oleic acid, linoleic acid, and α-linolenic acid).

The CSFII (4) is a large survey specifically designed to allow the estimation of food and nutrient intakes by the U.S. population. The CSFII and its predecessor surveys have been used by the U.S. Department of Agriculture for more than 70 yr to estimate food and nutrient intake and to evaluate the impact of proposed policy changes. Households and individuals were surveyed in all four seasons and on all days of the week. Approximately 16,000 individuals participated in the surveys. The CSFII 1998 added intake data from 5,559 children ages birth through 9 yr to the intake data collected from 4,253 children of the same ages participating in the CSFII 1994–96.

Because the CSFII database does not have *trans* FA compositions for the HSBO component of foods, we extrapolated from the USDA's database of the FA profiles, including *trans*-FA, of 214 foods (5). When this database did not provide the FA profiles for specific versions of HSBO used to prepare certain foods, these FA were estimated, using information from the fats/oils industry (Banks, D., personal communication). The methods used to assign these FA profiles to HSBO in foods are described in detail below.

Exponent's proprietary software program, FARE™(developed by Durango Software, LLC, Bethesda, MD), was used to

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Abbreviations: CSFII, Continuing Survey of Food Intakes by Individuals; H-LLSO, hydrogenated low linolenic acid soybean oil; HSBO, hydrogenated soybean oil; LLSO, low linolenic acid soybean oil.

analyze the CSFII data and estimate the intakes of total fat and FA from soybean oil in the diet (E_t) using the formula below. The intake of each food reported by each CSFII respondent $(Fc)_i$ (g/person/d) was multiplied by the soybean oil content of the food (SBO_f) (g/100 g food). Daily intakes of FA from soybean oil were calculated by multiplying the soybean oil intake by the assigned FA content of that soybean oil (FA_s), as described below. Mean and 90th percentile per capita dietary intakes for the total population and for eight age/sex groups were calculated as g/d:

$$E_t = \sum_i (\mathrm{Fc})_i (\mathrm{SBO}_f)_i (\mathrm{FA}_s)_i \quad [1]$$

where i = number of different food types consumed daily, Fc = amount of food consumed (g/d), SBO_f = soybean oil in food (g/100 g food), and FA_s = FA content of soybean oil (g/d).

Other publicly available statistical software programs such as SAS can be used to evaluate data from CSFII. The FARE™ software is currently used by the Center for Food Safety and Applied Nutrition (CFSAN) within the U.S. Food and Drug Administration.

We used a five-step process to determine the potential impact of substitution of LLSO for current versions of HSBO, as described below in detail.

Step One: Determination of current total fat intake and intake of five FA and trans FA. In using the CSFII database described above, the first step was to determine the mean and 90th percentile intakes of total fat, five FA, and total *trans* FA from all foods for the population and for eight age/sex groups.

Step Two: Determination of current total soybean oil intake and the categories of foods that are the major contributors to the dietary intake of soybean oil. The second step was to determine the mean and 90th percentile intakes of soybean oil. This was accomplished by identifying all foods in the CSFII surveys that contained soybean oil, whether hydrogenated or not, and multiplying the amount of soybean oil in the food by the amount of food consumed.

The categories of foods that contain hydrogenated (or partially hydrogenated) soybean oils were established by examining the FA composition of foods in the USDA *trans* FA database whose only added fat was provided by soybean oil and applying the following criteria: soybean oil-containing foods with *trans* FA concentrations greater than about 5% and α-linolenic acid levels less than about 4% were considered to be made with hydrogenated or partially hydrogenated soybean oil. The food categories of meat, poultry and fish mixtures, and egg dishes were divided into those prepared commercially or in restaurants vs. those prepared in the home. Foods in these categories that were prepared commercially or in restaurants were assumed to be prepared using HSBO, whereas those prepared in the home were assumed to be prepared with unhydrogenated, bottled soybean oil.

Step Three: Assignment of FA compositions to the soybean oils currently used in foods. In step three, a FA composition was assigned to all soybean oils currently used in foods. This step allowed the determination of baseline FA intake from soybean oils in all foods. The soybean oils used in foods can be unhydrogenated or hydrogenated to different degrees. Unhydrogenated soybean oil was assigned the FA composition presented in USDA's *trans* FA database, line #107, which describes a salad dressing made with unhydrogenated soybean oil. This FA profile was assumed to represent all unhydrogenated soybean oil (see Table 1). HSBO can have a number of FA compositions depending on the extent of hydrogenation. For example, light hydrogenation results in a small reduction in α-linolenic acid in soybean oil, whereas heavy hydrogenation results in virtually complete loss of α-linolenic acid and the creation of high proportions of *trans* FA. Changes in other FA in soybean oil also occur as a result of hydrogenation.

Since it is not possible to know the precise FA compositions for the various HSBO used in the several thousand foods made with soybean oil, we estimated a soybean oil FA composition for each category of food. We used the USDA *trans* FA database to assign FA compositions to the different types of soybean oils used in different food categories.

TABLE 1
FA Compositions of Low Linolenic Acid Soybean Oil Before and After Hydrogenation, and Comparator Soybean Oils

	% FA[a] (g/100 g fat)					
Soybean oil	16:0	18:0	18:1*c*	18:2n-6	18:3n-3	Total/*trans* FA
LLSO[b]	10.2	4.6	21.9	54.4	2.4	0.4
LLSO-hydrogenated[c]	10.2	11.3	40.0	3.1	0	30.3
USDA *trans* FA database, #107[d]	10.1	3.6	21.2	51.3	6.8	0.7
USDA *trans* FA database #107, hydrogenated[c]	10.1	10.2	38.7	3.1	0	31.4
All purpose, industrial, hydrogenated USDA/FNIC#04652[e]	10.8	12.1	30.2	4.4	0.2	32.8

[a]Values determined as % FA were converted to % fat by multiplying by 0.96.
[b]Low linolenic acid soybean oil (LLSO) was extracted and analyzed for FA composition using standard AOCS GC method #Ce 1b-89 (6).
[c]A mathematical modeling procedure was applied to derive the FA composition of the oil after theoretical hydrogenation (7).
[d]Item #107 in the USDA *trans* FA database (5) is a salad dressing made with unhydrogenated soybean oil as the only source of fat. Its FA composition is assumed to represent that of all unhydrogenated soybean oil used in this study.
[e]Reference 8; FNIC, Food and Nutrition Information Center.

TABLE 2
FA Assignments for the Hydrogenated Soybean Oils Used in Foods Included in the LLSO, H-LLSO Substitution

Food description	Corresponding USDA FA ID no.[a] or other reference	16:0	18:0	18:1*c*	18:2*c*	18:3*c*	Total *trans* FA
		% total fat[b]					
Baked goods: cakes, cookies, pies, pastries, pancakes, waffles, and other grain products	Hydrogenated #107 (see Table 1)	10.1	10.2	38.7	3.1	0	31.4
Meat/poultry/fish mixtures (commercial/restaurant)	Avg. of #81, 82, 83	11.1	9.3	30.2	15.1	0.8	20.1
Margarines							
Stick margarine	#88	10.7	6.7	30.0	15.0	1.5	30.4
Tub margarine	Avg. of #98, 99, 100	10.3	6.5	25.7	35.4	3.9	12.3
Potato products							
French fries (fast food and home)	Avg. of #81, 82, 83	11.1	9.3	30.2	15.1	0.8	20.1
Potato chips and puffs	(Banks, D., personal communication)	10.8	5.4	34.1	27.5	0.8	17.3
White potatoes, mashed, stuffed, puffs	#88	10.7	6.7	30.0	15.0	1.5	30.4
Creamy salad dressings	(Banks, D., personal communication)	10.2	5.1	25.9	34.6	2.7	14.4
Bread	Avg. of #7, 9, 47	11.9	5.8	24.7	35.9	3.0	5.2
Salty snacks from grain products	Avg. of #174, 175, 176, 177	11.2	10.4	30.7	8.9	0.3	31.1
Crackers	#27	9.6	7.3	31.5	4.6	0.3	36.1
Grain mixture dishes (burritos,enchiladas, tacos, other ethnic mixed dishes)	Hydrogenated #107 (Table 1)	10.1	10.2	38.7	3.1	0	31.4
Egg dishes (commercial/restaurant)	Avg. of #81, 82, 83	11.1	9.3	30.2	15.1	0.8	20.1

[a]Corresponds to food items as numbered in Reference 5.
[b]Values from the USDA *trans* FA database (5), expressed as g FA per g of food, were converted to g FA per 100 g fat by dividing the g FA per 100 g food by the g of total fat (expressed per 100 g food). H-LLSO, hydrogenated LLSO; for other abbreviations see Table 1.

The FA profiles for the soybean oils in 10 food categories that use hydrogenated oils (food categories in which LLSO was substituted for HSBO in subsequent analyses) are presented in Table 2. The FA profiles for the soybean oils used in other foods are presented in Table 3 (the remaining food categories for which no soybean oil substitutions were made). The sources of the data are presented in both tables. These FA compositions are based either on a single, representative food in the USDA *trans* FA database whose only added fat ingredient is soybean oil, or are an average of several similar foods whose only fat ingredients are soybean oil. For example, the FA composition of stick margarine is represented by item #88 in the USDA *trans* FA database, while averaging items #81, 82 and 83 represents the FA composition of French fries. In the case of grain mixture dishes, an HSBO similar to that used in baked goods was used. In the case of commercial/restaurant-prepared meat/poultry/fish mixtures and egg dishes, an HSBO like that used to prepare French fries was used. No examples of potato chips made only with soybean oil were identified in the USDA *trans* FA database, and the USDA reported wide variability in the composition of soybean oils used in creamy salad dressings. For these foods, information was obtained from the fats/oils industry (Banks, D., personal communication). In the case of baked goods, the FA composition of an HSBO, suitable for baked goods, was applied to all foods in that broad food category. Table 1 explains how this FA composition was derived.

Step Four: FA consumption from soybean oil. Once the FA profiles for the soybean oils currently used in all food categories in the CSFII database were assigned, an analysis was performed to provide the baseline intakes of five FA and *trans* FA from soybean oil for the total population at the mean and 90th percentile, and by age/sex groups.

Step Five: Determination of the impact of substitution of LLSO for HSBO in 13 categories of foods on intake of total trans FA and five FA. The final step was to analyze the impact on FA intake if LLSO were substituted for existing versions of HSBO. It was assumed that foods currently made with unhydrogenated soybean oils would continue to use existing varieties of soybean oils because manufacturers would be unwilling to pay a higher price for an oil whose additional stability is not needed. Thus, foods such as mayonnaise and oil and vinegar salad dressings were not included in the substitution analysis. Categories of foods that provided <0.2 g/d of soybean oil (<1% of the total) were not modified in the evaluation of the impact of substitution by LLSO since these categories would not contribute meaningful levels of individual FA to the overall intake.

Because the soybean oils used in baked goods typically must be heavily hydrogenated, LLSO *per se* would not be able to provide the needed functionality. However, since it is possible that LLSO could be hydrogenated to be used in baked goods, it was assumed that a hydrogenated version of LLSO

TABLE 3
FA Assignments for the Soybean Oils Used in Foods Excluded from the LLSO, H-LLSO Substitution

Food description	Corresponding USDA FA ID No.[a]	16:0	18:0	18:1*c*	18:2n-6	18:3n-3	Total *trans* FA
				(% total fat)			
Dairy (milk and milk dishes, and milk desserts)	107	10.1	3.6	21.2	51.3	6.8	0.7
Infant formula	107	10.1	3.6	21.2	51.3	6.8	0.7
Legume and nut dishes	107	10.1	3.6	21.2	51.3	6.8	0.7
Cereals (all ready-to-eat)	Hydrogenated #107 (see Table 1)	10.1	10.2	38.7	3.1	0	31.4
Frozen plate meals (grain as major ingredient)	100	9.9	6.3	22.3	40.1	5.5	10.1
Pasta (plain, cooked; macaroni, spaghetti, and rice noodles)	9	10.0	6.4	22.1	34.3	3.6	7.9
Oils and other fats:							
Oils, other	107	10.1	3.6	21.2	51.3	6.8	0.7
Cooking fats	98	10.1	6.6	27.6	30.7	2.7	16.1
Oil and vinegar-based salad dressings	Avg. of #107, 108, 110	9.9	3.7	21.7	37.4	6.8	0.5
Mayonnaise/Miracle Whip	105	9.9	3.6	24.9	45.4	5.9	4.3
Meat/poultry/fish mixtures, home prepared	107	10.1	3.6	21.2	51.3	6.8	0.7
Egg dishes, home prepared	107	10.1	3.6	21.2	51.3	6.8	0.7
Sugars and candies	107	10.1	3.6	21.2	51.3	6.8	0.7
Potato products (excluding those forms listed in Table 3)							
White potatoes, baked, boiled, not further specified	107	10.1	3.6	21.2	51.3	6.8	0.7
White potatoes (creamed, scalloped and au gratin)	107	10.1	3.6	21.2	51.3	6.8	0.7
Potato salads and recipes	105	9.9	3.6	24.9	45.4	5.9	4.3
Fruits	107	10.1	3.6	21.2	51.3	6.8	0.7

[a]Corresponds to food items as numbered in Reference 5. For abbreviations see Tables 1 and 2.

(H-LLSO) could be substituted for HSBO used in baked goods. The FA composition of LLSO is provided in Table 1. A hypothetical FA composition for H-LLSO was derived by applying a formula to achieve the same solid fat index as in regular HSBO for bakery applications (Table 1). The FA composition of unhydrogenated soybean oil as provided in the USDA FA database (line #107) can be compared with the estimated hydrogenated composition when this formula is applied to the original composition. This calculated composition of HSBO is very close to that for a hydrogenated, industrial soybean oil provided in the USDA Nutrient Composition Database (8).

Thus, in this analysis LLSO was substituted for existing versions of HSBO used in 9 food categories (other than baked goods) and H-LLSO for existing versions of HSBO used in baked goods. It was assumed that there would be 100% substitution of LLSO or H-LLSO for current versions of HSBO for the 10 categories of foods identified for substitution. These results were then compared against baseline values of intakes of FA from soybean oil currently used in all foods.

RESULTS

The baseline analysis indicated that per capita total fat intake by Americans was 72.8 g/d at the mean and 122.1 g/d at the 90th percentile (Table 4). Table 4 also shows the overall population intakes of individual FA, including *trans* FA. The population mean *trans* FA intake was 5.6 g/d, and at the 90th percentile, 10.4 g/d. Table 4 also shows the intakes of FA expressed as % energy. *Trans* FA provided 2.6% energy at the mean and 3.1% at the 90th percentile. The population mean intake of α-linolenic acid was 0.9 g/d (0.4% energy), and 1.6 g/d (0.5% energy) at the 90th percentile. Tables 5 and 6 provide the intakes of total fat and FA by age/sex groups in g/d. Data expressed as % energy by age/sex groups were essentially identical to those determined for the total population, as shown in Table 4, and are not shown in Tables 5 and 6.

To validate the results of our baseline analysis, we compared our results with those reported by Allison *et al.* (9). In that study, the CSFII 1989–1991 database was analyzed for total fat and FA, including *trans* FA. A comparison of our results with those of Allison *et al.* is shown in Table 7.

The small differences in FA intakes between the results of this study and those reported in the Allison *et al.* study could be due to small changes in the American diet between the time the two survey databases were collected, 1989–1991 and 1994–1998. Another possible explanation is that we applied a somewhat more hydrogenated nature to the soybean oils in our analysis vs. those used by Allison *et al*. This would account for

TABLE 4
Baseline Intakes[a] of Total Fat, Total *trans* FA, and Five FA by the U.S. Population

FA		Total U.S. population $n = 20,487$	
		Per capita intake mean	Per capita intake 90th percentile
Palmitic 16:0	g/d	13.1	22.3
	%en	6.0	6.6
Stearic 18:0	g/d	6.6	11.3
	%en	3.0	3.4
Oleic 18:1 (*cis*)	g/d	22.2	38.0
	%en	10.2	11.3
Linoleic 18:2 (*cis*)	g/d	9.8	17.9
	%en	4.5	5.3
α-Linolenic 18:3 (*cis*)	g/d	0.9	1.6
	%en	0.4	0.5
Total *trans* FA	g/d	5.6	10.4
	%en	2.6	3.1
Total fat intake	g/d	72.8	122.1
	%en	33.5	36.3

[a]Percent energy calculated by (g fat) × (9 kcal/g fat)/(mean or 90th percentile energy kcal).

the fact that we estimated slightly less α-linolenic and linoleic acids and slightly more *trans* FA than those reported by Allison *et al*. The lack of published data for the compositions of HSBO used in specific foods may also contribute to the minor differences observed in Table 5. This lack of published data requires estimations about the FA compositions of various soybean oils, and it is not surprising that different analyses provide somewhat varied results. Overall, there was good agreement between the results of this study and those of Allison *et al*.

Table 8 provides data on the mean and 90th percentile intake of soybean oil for the total population and by age/sex groups. Population mean intake was 21.8 g/d, and at the 90th percentile, 44.3 g/d. These data show that soybean oil contributes about one-third of the intake of total fat in the U.S. diet.

As shown in Table 9, the top 10 food categories containing HSBO account for 14.7 g of the 21.8 g/d mean total soybean oil consumption. These 10 food categories were the targets of LLSO or H-LLSO substitution in subsequent analyses.

Table 10 shows the mean and 90th percentile per capita intakes of FA from soybean oil by the U.S. population. The analysis was repeated for age/sex groups, and the results by age for males and females are presented in Tables 11 and 12, respectively. The mean intake of *trans* FA from soybean oil was 3.6 g/d (1.7% energy), and at the 90th percentile was 8.0 g/d (2.4% energy). These data form the baseline reference against which the effects of substitution of LLSO on intake of FA from soybean oil are measured.

Tables 13–15 provide data on the mean and 90th percentile per capita intakes of FA from soybean for the U.S. population and by age/sex groups after LLSO was substituted for HSBO in nine categories of foods and H-LLSO was substituted for HSBO in baked goods. These substitutions resulted in a decrease in mean *trans* FA intake from soybean oil from 3.6 to 1.1 g/d (1.7 to 0.5% energy), and at the 90th percentile, from 8.0 to 3.4 g/d (2.4 to 1.0% energy) (Tables 10 and 13). Tables 11 and 14 show the impact of this substitution on the FA intakes of males, and Tables 12 and 15 show the impact on that of females. Males ages 9–19 and 20–49 yr at the 90th percentile had baseline *trans* FA intakes from soybean oil of 10.5 g/d. These are the highest levels of *trans* FA intakes from soybean oil observed in this analysis. After substitution with LLSO and H-LLSO, *trans* FA intakes from soybean oil in these two age/sex groups decreased to 3.9–4.1 g/d, about a 62% reduction. Females ages 9–19 and 29–49 yr at the 90th percentile had baseline *trans* FA intakes from soybean oil of 7.9 and 7.1 g/d, respectively. After LLSO and H-LLSO substitution, *trans* FA intakes from soybean oil decreased to 3.4 and 2.9 g/d for females in the 9–19 and 20–49 yr age/sex groups, respectively, or reductions of about 57%.

Intake of α-linolenic acid, an essential ω-3 FA, from soybean oil did not decrease at the mean or 90th percentile intakes as a result of substitution of LLSO for HSBO in the nine categories and H-LLSO in baked goods. The mean baseline intake of α-linolenic acid from soybean oil was 0.6 g/d, and at the 90th percentile was 1.3 g/d. After LLSO and H-LLSO substitution as described above, there were 0.1 and 0.2 g/d increases at the mean and 90th percentile, respectively (Table 10 vs. 13).

TABLE 5
Baseline Intakes[a] of Total Fat, Total *trans* FA Intake, and Five FA by Male U.S. Population Groups

FA	Males 1–8 yr $n = 3883$		Males 9–19 yr $n = 1082$		Males 20–49 yr $n = 2358$		Males 50+ yr $n = 2393$	
	Mean	90th	Mean	90th	Mean	90th	Mean	90th
	(g/d)							
Palmitic 16:0	11.6	17.7	17.4	27.5	17.8	28.2	13.7	22.7
Stearic 18:0	5.8	9.1	8.8	14.1	8.9	14.4	7.1	11.8
Oleic 18:1 (*cis*)	17.8	28.2	28.3	45.5	30.3	48.0	24.2	39.7
Linoleic 18:2 (*cis*)	6.6	11.5	11.6	20.4	13.4	22.8	10.9	19.2
α-Linolenic 18:3 (*cis*)	0.5	0.9	1.0	1.7	1.2	2.1	1.0	1.8
Total *trans* FA	4.8	8.1	7.5	13.0	7.3	12.9	6.0	10.7
Total fat intake	60.7	92.0	93.3	146.9	98.2	153.2	78.0	126.4

[a]For footnote see Table 4.

TABLE 6
Baseline Intakes[a] of Total Fat, Total *trans* FA Intake, and Five FA by Female U.S. Population Groups

	Females 1–8 yr *n* = 3741		Females 9–19 years *n* = 1092		Females 20–49 yr *n* = 2319		Females 50+ yr *n* = 2253	
FA	Mean	90th	Mean	90th	Mean	90th	Mean	90th
				(g/d)				
Palmitic 16:0	10.6	16.2	12.2	19.3	11.0	18.2	9.3	15.1
Stearic 18:0	5.3	8.1	6.2	10.0	5.6	9.2	4.8	7.9
Oleic 18:1 (*cis*)	16.3	25.2	20.2	31.6	19.1	31.9	16.4	26.4
Linoleic 18:2 (*cis*)	6.2	10.4	8.5	14.0	9.0	16.5	8.0	14.1
α-Linolenic 18:3 (*cis*)	0.5	0.9	0.7	1.2	0.8	1.5	0.7	1.4
Total *trans* FA	4.4	7.2	5.7	9.7	4.9	8.6	4.2	7.3
Total fat intake	55.6	82.8	66.2	103.1	62.3	100.2	53.9	84.8

[a]For footnote see Table 4.

All age/sex groups at both mean and 90th percentile intakes showed either no change or small increases in intakes of α-linolenic acid from soybean oil as a result of substitution of LLSO and H-LLSO for current versions of HSBO (Table 11 vs. 14 and Table 12 vs. 15).

Substitutions of LLSO and H-LLSO caused very small changes in the remaining FA, generally less than 2 g/d, with the exception of intake of linoleic acid from soybean oil, which approximately doubled at both the U.S. population mean and 90th percentile intakes (Table 10 vs. 13). These same results also were observed for all age/sex groups at both the mean and 90th percentiles of intake (Table 11 vs. 14; Table 12 vs. 15).

Figure 1 shows the overall dietary impact of substitution of LLSO or H-LLSO for HSBO in 10 food categories, expressed as % energy. Substitution of LLSO or H-LLSO resulted in an overall reduction in *trans* FA intake of about 1.2% energy (2.5 g/d) at the mean and about 1.4% energy (4.6 g/d) at the 90th percentile. Thus, postsubstitution, the total *trans* FA intake decreased from 2.6 to 1.4% energy at the mean, and from 3.1 to 1.7% energy at the 90th percentile. Changes in intakes of other FA were small, within 1% energy, with the exception of linoleic acid, which could increase by about 2–2.5% energy. This increase in linoleic acid intake results from the fact that LLSO has a linoleic acid content of about 54% whereas the linoleic acid content of partially HSBO is reduced due to the hydrogenation of linoleic acid to oleic and/or stearic acids as well as to *trans* FA.

TABLE 7
Comparison of the Results of Total Fat and FA Intake from the Current Analysis vs. Those of Allison *et al.* (9)

	Mean per capita daily intakes of total fat and FA by Americans	
	Current analysis (g/d)	Allison *et al.* (9) (g/d)
Total fat	72.8	70.7
Palmitic acid	13.1	13.6
Stearic acid	6.6	6.4
Oleic acid (*cis* isomer)	22.2	19.6
Linoleic acid (*cis* isomer)	9.8	12.1
α-Linolenic acid (*cis* isomer)	0.9	1.2
Total *trans* FA	5.6	5.3

DISCUSSION

This analysis shows that substitution of LLSO and H-LLSO for HSBO can result in meaningful decreases in consumer intakes of *trans* FA from soybean oil with no reduction in intake of α-linolenic acid, an essential ω-3 FA. HSBO has been a major source of *trans* FA in the diet. As estimated in this study, HSBO provided 3.6 g/d *trans* FA at the mean intake (1.7% energy), and 8.0 g/d at the 90th percentile (2.4% energy). The total diet provided 5.6 g/d *trans* FA at the mean and 10.4 g/d at the 90th percentile (2.6 and 3.1% energy, respectively). Other sources of *trans* FA in the diet include other hydrogenated vegetable oils and animal products. However, since soybean oil provides about 80% of the total intake of vegetable oils in the U.S. diet, and since the *trans* FA in animal products are inherent, it is reasonable to target *trans* FA intake reduction at HSBO. This substitution analysis shows that substitution of LLSO for HSBO in nine food categories and use of H-LLSO in the baked goods category can result in a *trans* FA content of the diet of 1.4% energy at the mean and 1.7% energy at the 90th percentile. These

TABLE 8
Baseline Intakes[a] of Soybean Oil

	Soybean oil intake (g/d)	
Population	Mean	90th percentile
U.S. (total population)	21.8	44.3
Males		
1–8 yr	15.7	30.7
9–19 yr	27.7	54.0
20–49 yr	29.1	57.1
50+ yr	23.2	46.1
Females		
1–8 yr	14.6	28.6
9–19 yr	20.6	38.6
20–49 y	19.4	40.1
50+ yr	17.0	34.5

[a]For footnote see Table 4.

TABLE 9
Foods Made with Partially Hydrogenated or Hydrogenated Soybean Oils and Their Contribution to Soybean Oil Intake

Food categories	Per capita mean soybean oil intake (g/d)
Baked goods:	
Cakes	0.7
Cookies	0.8
Pies	0.5
Pastries	0.9
Pancakes, waffles, French toast, other grain products	0.2
Meat, poultry, fish mixtures: commercially prepared and restaurant	0.3
Margarines:	
Stick margarine	0.4
Tub margarine	0.7
Potato products:	
French fries	1.7
Potato chips, puffs	1.0
Mashed, stuffed, puffs	0.4
Creamy salad dressings	1.0
Bread	2.4
Salty snacks from grain products	1.2
Crackers (sweet, nonsweet, low sodium)	0.4
Grain mixture dishes	1.9
Eggs (commercial and restaurant)	0.2
Sum	14.7

TABLE 10
Per Capita Baseline Intake[a] of FA from Soybean Oil by the Total Population

FA		Per capita intake mean	Per capita intake 90th percentile
Palmitic 16:0	g/d	2.3	4.7
	%en	1.1	1.4
Stearic 18:0	g/d	1.5	3.2
	%en	0.7	1.0
Oleic 18:1	g/d	6.3	12.9
	%en	2.9	3.8
Linoleic 18:2 (*cis*)	g/d	5.5	12.0
	%en	2.5	3.6
α-Linolenic 18:3 (*cis*)	g/d	0.6	1.3
	%en	0.3	0.4
Total *trans* FA	g/d	3.6	8.0
	%en	1.7	2.4

[a]Percent energy calculated as (g fat) × (9 kcal/g fat)/(mean or 90th percentile energy kcal).

levels approach those recommended by the Dietary Guidelines Advisory Committee (3).

Soybean oil is among the richest and most abundant sources of α-linolenic acid in the American diet (10). This study showed that soybean oil contributed 0.6 g/d on average (67%), and 1.3 g/d at the 90th percentile (81%), to total α-linolenic acid intake. Canola and flax seed oil have higher concentrations of α-linolenic acid than soybean oil, but the consumption of these oils by Americans is small. Nuts such as walnuts are also rich sources, but again, intakes are low. This analysis has shown that substitution of LLSO or H-LLSO for HSBO in 10 food applications does not result in a lowering in the dietary intake of α-linolenic acid. Although the α-linolenic acid content of LLSO is 2.4%, lower than the level in unhydrogenated soybean oil (about 7%), LLSO is intended to substitute for a variety of HSBO, which are as low in α-linolenic acid as LLSO, if not lower. As shown in Table 2, the estimated α-lino-

TABLE 11
Baseline Intake of FA from Soybean Oil by Males

	Males 1–8 yr		Males 9–19 yr		Males 20–49 yr		Males 50+ yr	
FA	Mean	90th	Mean	90th	Mean	90th	Mean	90th
				(g/d)				
Palmitic 16:0	1.7	3.2	3.0	5.7	3.1	6.1	2.4	4.8
Stearic 18:0	1.2	2.5	2.1	4.1	2.0	4.0	1.5	3.2
Oleic 18:1 (*cis*)	4.8	9.4	8.3	15.9	8.4	16.6	6.5	13.2
Linoleic 18:2 (*cis*)	3.2	6.8	6.1	13.1	7.4	15.6	6.4	13.7
α-Linolenic 18:3 (*cis*)	0.3	0.7	0.6	1.3	0.8	1.7	0.7	1.6
Total *trans* FA	3.1	6.5	5.1	10.5	4.7	10.5	3.4	7.8

TABLE 12
Baseline Intake of FA from Soybean Oil by Females

	Females 1–8 yr		Females 9–19 yr		Females 20–49 yr		Females 50+ yr	
FA	Mean	90th	Mean	90th	Mean	90th	Mean	90th
				(g/d)				
Palmitic 16:0	1.6	3.0	2.1	4.1	2.1	4.2	1.8	3.6
Stearic 18:0	1.1	2.2	1.6	3.0	1.3	2.8	1.1	2.3
Oleic 18:1 (*cis*)	4.4	8.7	6.2	11.9	5.6	11.5	4.7	9.6
Linoleic 18:2 (*cis*)	3.1	6.4	4.5	9.1	5.0	11.0	4.9	10.5
α-Linolenic 18:3 (*cis*)	0.3	0.7	0.4	0.9	0.5	1.2	0.5	1.2
Total *trans* FA	2.9	6.0	3.9	7.9	3.1	7.1	2.4	5.6

TABLE 13
Intake of FA from Soybean Oil by the Total Population After Substitution of LLSO and H-LLSO for Regular Soybean Oils in 10 Categories of Foods[a]

FA		Per capita intake mean	Per capita intake 90th percentile
Palmitic 16:0	g/d	2.2	4.5
	%en	1.0	1.3
Stearic 18:0	g/d	1.2	2.4
	%en	0.6	0.7
Oleic 18:1	g/d	5.4	11.1
	%en	2.5	3.3
Linoleic 18:2 (*cis*)	g/d	9.8	20.4
	%en	4.5	6.1
α-Linolenic 18:3 (*cis*)	g/d	0.7	1.5
	%en	0.3	0.4
Total *trans* FA	g/d	1.1	3.4
	%en	0.5	1.0

[a]Percent energy calculated by (g fat) × (9 kcal/g fat)/(mean or 90th percentile energy kcal). For abbreviations see Tables 1 and 2.

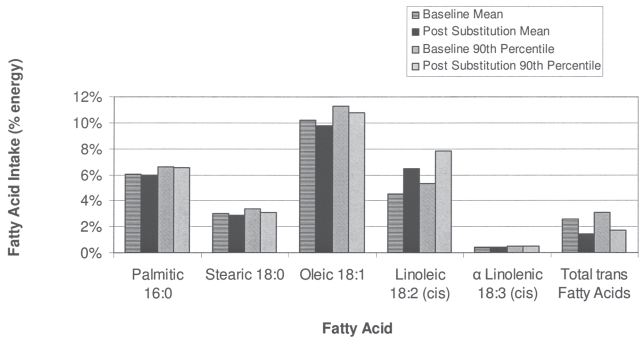

FIG. 1. Total dietary intake of FA: baseline vs. post-LLSO, H-LLSO substitution and U.S. per capita mean and 90th percentile (% energy). LLSO, low linolenic acid soybean oil; H-LLSO, hydrogenated LLSO.

lenic acid composition of the HSBO used in processed foods ranges from zero to 3.9%. Thus, when the impact of substitution is considered across this range of HSBO, it becomes clear why LLSO had no impact on intake of α-linolenic acid.

Changes in intakes of other FA associated with substitution of LLSO and H-LLSO for HSBO were within the range of normal and nutritious dietary intakes. It is important to note that use of LLSO or H-LLSO did not raise the intake of saturated FA but did raise the intake of linoleic acid by a few grams per day on average. Thus, the overall combination of effects on FA intake, i.e., a reduction in *trans* FA, no increase in saturated FA, an increase in linoleic acid, and no decrease in α-linolenic acid, is consistent with the 2005 Dietary Guidelines for Americans (3).

This analysis assumed 100% market penetration of LLSO for current versions of HSBO in nine food categories and of H-LLSO in baked goods. This assumption allows one to see the extremes of substitution on FA intakes. It is likely that the marketplace will use LLSO and H-LLSO at less than 100% penetration and use other substitutes for HSBO besides LLSO and H-LLSO; thus the actual impact on FA intakes will likely be somewhat different from that estimated here. As more options to replace hy-

TABLE 14
Intake of FA from Soybean Oil by Males After Substitution of LLSO and H-LLSO for Regular Soybean Oils in 10 Categories of Foods[a]

	Males 1–8 yr		Males 9–19 yr		Males 20–49 yr		Males 50+ yr	
FA	Mean	90th	Mean	90th	Mean	90th	Mean	90th
				(g/d)				
Palmitic 16:0	1.6	3.1	2.8	5.5	3.0	5.8	2.4	4.7
Stearic 18:0	0.9	1.8	1.5	3.0	1.5	3.1	1.3	2.6
Oleic 18:1 (*cis*)	4.0	7.9	6.9	13.4	7.0	13.9	5.8	11.8
Linoleic 18:2 (*cis*)	6.8	13.7	12.5	24.8	13.5	27.2	10.1	20.8
α-Linolenic 18:3 (*cis*)	0.4	0.8	0.8	1.6	0.9	1.9	0.8	1.6
Total *trans* FA	1.0	2.8	1.5	4.1	1.3	3.9	1.4	3.9

[a]For abbreviations see Tables 1 and 2.

TABLE 15
Intake of FA from Soybean Oil by Females After Substitution of LLSO and H-LLSO for Regular Soybean Oils in 10 Categories of Foods[a]

	Females 1–8 yr		Females 9–19 yr		Females 20–49 yr		Females 50+ yr	
FA	Mean	90th	Mean	90th	Mean	90th	Mean	90th
				(g/d)				
Palmitic 16:0	1.5	2.9	2.1	3.9	2.0	4.1	1.7	3.5
Stearic 18:0	0.8	1.7	1.1	2.3	1.0	2.2	0.9	1.9
Oleic 18:1 (*cis*)	3.7	7.3	5.1	9.9	4.8	10.0	4.2	8.7
Linoleic 18:2 (*cis*)	6.3	12.8	9.2	18.2	8.8	18.2	7.5	15.3
α-Linolenic 18:3 (*cis*)	0.4	0.8	0.6	1.2	0.6	1.3	0.6	1.3
Total *trans* FA	0.9	2.6	1.2	3.4	1.0	2.9	1.0	2.9

[a]For abbreviations see Tables 1 and 2.

drogenated oils become available on the market, it will be important to estimate their potential impact on the FA composition of the diet.

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A High-Saturated Fat Diet Enriched with Phytosterol and Pectin Affects the Fatty Acid Profile in Guinea Pigs

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ABSTRACT: This paper presents the results of a study whose aim was to test the effects of several doses of pectin and phytosterols on the body weight gain and the FA content in female guinea pigs. The treatments resulted from supplementing with pectin and plant sterol a guinea pig diet (rich in saturated FA), following a 3×3 factorial design, with three levels of pectin (0, 3.67 and 6.93%) and three levels of phytosterols (0, 1.37, and 2.45%). Seventy-two female Dunkin Hartley guinea pigs were randomly assigned to the treatment groups (8 animals/group), the duration of the treatment being 4 wk. Pectin dietary intake led to a significant increase in body weight ($P < 0.001$), food consumption ($P = 0.025$), and feed efficiency ($P < 0.001$), but no influence of phytosterols on weight gain or food consumption was detected. We found a significant negative effect of the addition of phytosterols on lauric, myristic, and palmitic acid contents in feces, and a positive effect on their concentration in plasma and liver, but no significant effect on stearic acid content. Apparent FA absorption was assessed by calculating the ratio of FA in feces and diets that the absorption of the different FA could be compared, and the negative effect of phytosterol supplementation on these ratios, especially for lauric and myristic acids, was established.

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During the past several decades, reduction in fat intake has been the main focus of dietary recommendations. In particular, it has been recommended to "choose a diet low in saturated fat and cholesterol and moderate in total fat" to prevent coronary heart disease (CHD) (1,2). Thus, there is a shift going on in nutrition, from the elimination of nutrient deficiencies, toward the possibility of improving health and quality of life. This awareness has lead to the development of functional foods, claiming beneficial effects for consumers. Several studies have shown that the ingestion of bioactive substances, such as soluble fibers (guar gum, psyllium, pectin, and oat products) and phytosterols (stanols and sterols) may have a positive lipidemic effect. As a consequence, they have been incorporated into some foods to reduce LDL-cholesterol levels and therefore prevent the development of CHD (3,4).

The cholesterol-lowering efficacy of plant sterol and stanol esters in food products has been confirmed in a great number of studies (5–10). Several mechanisms have been suggested to explain the action of phytosterols on lipid metabolism. For instance, β-sitosterol and sitostanol reduce plasma total-cholesterol levels through several mechanisms, such as competitively blocking cholesterol absorption from the intestinal lumen (11,12), displacing cholesterol from bile salt micelles (13), increasing bile salt excretion (14), or hindering the cholesterol esterification rate in the intestinal mucosa (15,16). Daily consumption of moderate quantities of phytosterols also has been shown to reduce plasma total-cholesterol by 5 to 13% and LDL-cholesterol by 7 to 16%, in both hyper- (17,18) and normocholesterolemic (19) individuals, without affecting HDL-cholesterol or TAG concentrations.

On the other hand, it is well known that including in the diet soluble viscous fibers such as pectin or guar gum as well as nonfermentable viscous fibers such as psyllium helps to lower plasma cholesterol concentrations (20–24). The soluble fiber action is mainly effected by binding bile acids, thus provoking a reduction in bile acid reabsorption of the small intestine.

Nevertheless, a separate examination of the effect of both phytosterols and pectin on the different FA is needed because there are different pathways of absorption for short-chain and long-chain FA. As early as 1935, Hughes and Wimmer (25) suggested that FA may be absorbed by different routes, according to their chain length. Since then, a number of studies (26) have established the role of the lymph and portal blood absorption pathways.

Finally, it is important to note that the consumption of vegetable shortenings is increasing because of their use in ready-to-eat foods (27) and in deep-fat frying. This is an undesirable development, because the intake of *trans* FA and saturated FA (SFA), which are two of the most important components of Western diets (28,29), is related to an increased risk of cardiovascular disease (1,2).

Therefore, the aim of the present study was to examine the effects of several doses of pectin and phytosterols on FA content in female guinea pigs fed a diet rich in SFA and cholesterol. The comparison of fecal and dietary levels allows us to examine the effects of this type of supplementation to a diet enriched with SFA on the apparent absorption (30–33) of the different FA. It has been reported (34,35) that results regarding apparent absorption were similar to those obtained using stable isotopes. Moreover, it is also important to determine the FA content in some biological tissues, such as liver and plasma.

The guinea pig was chosen as the animal model owing to its similarities with humans (LDL/HDL ratio, distribution of

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Abbreviations: CHD, coronary heart disease; MUFA, monounsaturated FA; SFA, saturated FA.

cholesterol pools, and activities of main enzymes regulating cholesterol metabolism), and because guinea pigs respond to dietary treatments by changing plasma cholesterol concentrations in the same way as humans do (36).

MATERIALS AND METHODS

Materials. All FAME standards (12:0, 14:0, 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 19:0, and 21:0), with purity greater than 99%, were purchased from Sigma Chemical Co. (St. Louis, MO). The identification of FAME was made with FAME Mix, C_4–C_{24} (Supelco, Bellefonte, PA).

Boron trifluoride in methanol (1.7 mol/L) and *n*-hexane were purchased from Merck (Darmstadt, Germany), and sodium chloride and anhydrous sodium sulfate from Panreac (Barcelona, Spain). Sodium methylate (0.5 mol/L) was prepared by dissolving 17 g of sodium in 1 L of dry methanol.

Diets. Nine isocaloric diets, designed to meet all the nutritional requirements for guinea pigs, were prepared by Mucedola SRL (Settimo Milanese, Italy). The composition of all diets was similar, except for the content of pectin and phytosterols. Pectin was purchased from CP Kelco (Atlanta, GA). We used GENU-type Freeze pectin, which is a high (methyl) ester pectin (containing more than 50% of the carboxylic acid as methyl esters). This kind of pectin is a mixture of high-ester pectin extracted from citrus peel and α-amylolytic enzyme complex, which is standardized by addition of sucrose. Plant sterols were esterified with unsaturated FA (Raisio, Helsinki, Finland).

The treatments resulted from the addition of pectin and plant sterols, following a 3×3 factorial design, with three levels of pectin, denoted by PE0 (0%), PE1 (3.67%), and PE2 (6.93%), and three levels of phytosterols, denoted by PH0 (0%), PH1 (1.37%), and PH2 (2.45%). The chemical composition of the diets is shown in Table 1. The FA content in each diet was determined by the method proposed by Lopez-Lopez *et al.* (37).

Table 2 shows the content (μg/mg) of seven FA (12:0, 14:0, 16:0, 18:0, 18:1n-9, 18:2n-6, and 18:3n-3) in the nine diets. The presentation of results in this article is restricted to these seven FA, selected by their accumulation pattern and the differences observed in feces. The addition of pectin did not affect the total FA content but, since phytosterols were esterified with unsaturated FA, the FA profile was modified.

The SFA fraction ranged from 84 to 96% of the total FA content of the diet. The monounsaturated FA (MUFA) fraction was much smaller, but the most variable, ranging from 2 to 11% owing to the variations in the oleic acid content (Table 2). The PUFA fraction fluctuated from 1 to 5% of the total FA content. The main SFA was lauric acid (40–47%), followed by myristic acid (about 16%) and palmitic and stearic acids, with similar levels (9–10%). Also relevant was the presence of short-chain SFA, essentially 8:0 and 10:0, which accounted for 10% of the total FA content; however these are not reported in Table *2*, since their content in feces was below 1%. Oleic acid was the main unsaturated FA, presenting also the highest variation across diets (2–10%).

Animals. Seventy-two female Dunkin Hartley guinea pigs, supplied by Harlan Interfauna Ibérica (Barcelona, Spain) and weighing 300–350 g, were randomly assigned to the treatment groups (8 animals/group). The duration of the treatment was 4 wk. The guinea pigs were housed two per cage in a light-cycle room (light from 0800 to 2000), with free access to feed and water. They were killed by heart puncture after halothane anesthesia. Blood samples were obtained by means of an intracardiac extraction, and liver was extracted after the sacrifice and homogenized. Feces were collected with a 2-d periodicity and freeze-dried immediately. All procedures were approved by the Animal Care and Use Committee of the University of Barcelona.

FA analysis. The FA content in feces and in diets was determined with the method of Lopez-Lopez *et al.* (37). To determine the FA concentration in liver and plasma, we used a

TABLE 1
Composition of Diets[a] Fed to Guinea Pigs

Treatment	Pectin	Phytosterols[b]	Protein[c]	Fat[c]	Sugars[c]	Insoluble fiber[c]	Mineral and vitamin mix[d]
PE0/PH0	0	0	18.3	15.9	38.4	12.7	6.5
PE0/PH1	0	1.37	18.5	17.6	40.0	11.9	6.7
PE0/PH2	0	2.45	17.7	19.6	39.4	11.5	6.5
PE1/PH0	3.7	0	18.6	15.9	35.3	12.5	6.7
PE1/PH1	3.7	1.37	18.9	17.4	36.2	12.5	6.7
PE1/PH2	3.7	2.45	18.0	19.7	33.6	12.5	6.7
PE2/PH0	6.9	0	18.7	15.6	33.1	12.4	6.7
PE2/PH1	6.9	1.37	18.9	17.4	30.5	11.9	6.8
PE2/PH2	6.9	2.45	18.1	19.5	29.2	11.8	6.7

[a]All diets had been enriched with 0.33% of cholesterol.
[b]Added on top of the basal diet. The plant sterol ester mixture was mixed into the feed and comprised 6.4% brassicasterol, 24.9% campesterol, 1.0% campestanol, 18.5% stigmasterol, 45.6% β-sitosterol, 1.8% sitostanol, 0.9% Δ5-avenasterol, and 1.0% other sterols. PE0, PE1, PE2, pectin levels of 0, 3.67, and 6.93%, respectively; PH0, PH1, and PH2, phytosterol levels of 0, 1.37, and 2.45%, respectively.
[c]Protein content was casein; fat was hydrogenated coconut oil; sugars were corn starch, dextrose, and sucrose; and insoluble fiber was cellulose.
[d]Vitamin and mineral mixes were adjusted to meet National Research Council requirements for guinea pigs. A detailed composition of the vitamin and mineral mix has been reported by Krause and Newton (36).

TABLE 2
Content of the Main FA in the Diets

		FA (μg/mg)						
Pectin	Phytosterol	12:0	14:0	16:0	18:0	18:1n-9	18:2n-6	18:3n-3
PE0	PH0	54.61	20.62	11.35	12.63	2.98	1.27	ND[a]
PE0	PH1	41.98	15.88	9.35	10.00	6.45	2.32	0.60
PE0	PH2	47.26	17.87	10.53	11.38	11.81	4.20	1.27
PE1	PH0	49.61	22.23	12.12	13.30	3.65	1.42	ND
PE1	PH1	44.82	16.73	9.66	10.13	6.92	2.48	0.61
PE1	PH2	48.04	18.12	11.04	11.31	12.14	4.26	1.23
PE2	PH0	60.76	22.52	12.41	13.46	3.53	1.37	ND
PE2	PH1	44.32	16.75	9.99	10.44	6.82	2.30	0.61
PE2	PH2	45.34	17.19	10.30	10.98	11.20	3.76	1.16

[a]ND, not detected. For other abbreviations see Table 1.

method described elsewhere (38) but with some modifications to adapt it to our samples, followed by a double methylation to obtain FAME.

(i) Determination of FA content in feces. First, 25 μL of internal standard (21:0, 466 μg/mL) was added to 50 mg of freeze-dried fecal homogenate. Then, a small magnetic stirring bar was added and the sample was saponified with 1 mL of sodium methylate (0.5 mol/L) and heated in a water bath at 90°C for 15 min. The tubes were removed from the water bath and cooled in another water bath at room temperature. Then, 1 mL of boron trifluoride/methanol (1.7 mol/L) was added. Next, the tubes were placed in the water bath at 90°C for 15 min and subsequently cooled as before; 400 μL of *n*-hexane was then added. The tubes were shaken, and 1 mL of a saturated solution of sodium chloride in distilled water was added, followed by centrifugation. The clear *n*-hexane top layer, containing the FAME, was transferred to another tube, and a small quantity of anhydrous sodium sulfate was added. The tubes were stored at −20°C until the contents were injected into the gas chromatograph.

(ii) Determination of FA content in liver. Hepatic homogenate (between 0.8 and 1.0 g) was weighed into a 32 × 210 mm tube, and 200 μL of internal standard (21:0, 466 μg/mL) was added. Just before homogenization, 20 mL of chloroform/methanol (2:1, vol/vol) was added. The tube contents were homogenized (30 s, 12,000 rpm) in a Polytron PT 3000 (Kinematica, Lucerne, Switzerland), keeping the tubes in an ice bath. The extract was filtered through a Whatman No. 1 filter paper into a 50 mL screw-capped tube, and the residue was re-extracted twice (10 mL) with the same solvent. Next, 10 mL of water was added to the tube and, after centrifugation (20 min, 750 × *g*), the chloroform phase was filtered through anhydrous sodium sulfate (Whatman No. 1 filter paper), which was then washed twice with 10 mL of chloroform. The lipid extract obtained was dried in a vacuum rotary evaporator at 30°C, and re-extracted five times with 2 mL of diethyl ether. The tubes were dried first by a slight nitrogen stream and then by keeping the flask in a vacuum desiccator at 10 mmHg overnight.

After lipid extraction, a small magnetic stirring bar was added, and the samples were saponified with 2.5 mL of sodium methylate (0.5 mol/L) and heated in a water bath (90°C, 20 min). After that, the tubes were cooled in a water bath at room temperature, and 3 mL of boron trifluoride/methanol (1.7 mol/L) was added. Next, they were placed in the water bath at 90°C for 20 min, subsequently cooled as before, and 2 mL of *n*-hexane was added. The tubes were shaken, and 3 mL of a saturated solution of sodium chloride in distilled water was added, followed by centrifugation. The clear *n*-hexane top layer, containing the FAME, was transferred to another tube, and a small quantity of anhydrous sodium sulfate was added. The tubes were stored at −20°C until the contents were injected into the gas chromatograph.

(iii) Determination of FA content in plasma. Plasma (100 μL) was added to a tube containing 25 μL of internal standard (19:0, 996 μg/mL). Then, 3 mL of chloroform/methanol (2:1, vol/vol) was added and mixed. After that, 0.75 mL of 0.8% aqueous NaCl was added, mixing again. After centrifugation (7 min, 1025 × *g*), the chloroform phase was transferred to another tube, and the aqueous one was washed with 3 mL of chloroform. The lipid extract obtained was dried with a slight nitrogen stream, keeping the flask in a vacuum desiccator at 10 mmHg overnight. The double methylation was done in the same way as in hepatic samples, but instead of using 2.5 mL of sodium methylate and 3 mL of boron trifluoride/methanol, 1 mL of each was used. FAME were obtained by adding 400 μL of *n*-hexane to tubes, followed by 1 mL of a saturated solution of sodium chloride in distilled water. The clear *n*-hexane top layer, containing FAME, was transferred, adding a small quantity of anhydrous sodium sulfate. The tubes were stored at −20°C until injection into the gas chromatograph.

GC conditions. FAME were analyzed with an Agilent 4890D gas chromatograph, equipped with an FID. The separation of FAME was done in a fused-silica column (60 m × 0.20 mm i.d., 0.2 μm). The split-splitless injector was used in split mode with a ratio of 1:30. Injector and detector temperatures were kept at 270 and 300°C, respectively. For feces analysis the oven temperature was programmed as follows: an initial period of 5 min at 100°C, followed by an increase of 4°C/min, up to 235°C, and a final period of 2 min at this temperature. In liver samples the oven program started with a period of 5 min at 140°C, followed by an increase of 1.8°C/min until 180°C, followed by another increase of 7.5°C/min until 235°C. Finally, for FAME determination in plasma samples, the initial temperature was 170°C (11 min), being for the rest the same as in liver samples.

TABLE 3
Food Consumption and Final Body Weight

Pectin	Phytosterol	Food consumption[a]	Body weight[b]
PE0	PH0	35.30 ± 7.84	374.55 ± 13.11
PE0	PH1	32.56 ± 1.33	364.75 ± 30.20
PE0	PH2	33.59 ± 5.78	368.41 ± 25.61
PE1	PH0	32.27 ± 4.55	400.97 ± 34.97
PE1	PH1	35.69 ± 7.18	400.04 ± 52.80
PE1	PH2	33.66 ± 14.47	397.09 ± 24.19
PE2	PH0	29.25 ± 1.49	388.85 ± 66.68
PE2	PH1	28.70 ± 3.35	412.44 ± 17.00
PE2	PH2	27.99 ± 2.75	412.70 ± 33.04

[a]Mean of consumption (g/d/animal) ± SD.
[b]Mean of body weight (g) ± SD. For abbreviations see Table 1.

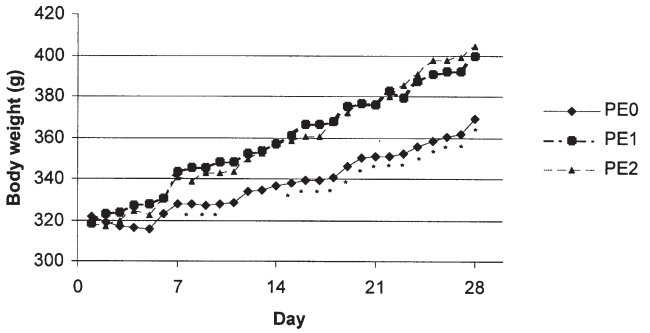

FIG. 1. Mean body weight curves for different pectin levels. PE0, PE1, and PE2, pectin levels of 0, 3.67, and 6.93%, respectively. An asterisk (*) means significant differences among diets ($P < 0.05$).

Helium was used as the carrier gas. Chromatographic peaks were identified by comparing the retention times with those of known standards and by cochromatography. FAME quantification was performed by the internal standard addition method.

Statistical analysis. The effects of the treatments on body weight gain and FA content were tested by two-way ANOVA with the amount of pectin and phytosterols as factors. Since an assumption of homogeneity of variance in the case of food consumption (Table 3), where coefficients of variance range from 4 to 43%, was unrealistic, we used the Kruskal–Wallis test to assign a significance level to the pectin supplementation effect.

On the other hand, since the FA profile of the diets changes with phytosterol supplementation, as a result of esterification, the phytosterol effect can be confounded with a diet effect, especially for the MUFA and PUFA. To account for these diet effects, we performed the statistical analysis, for the different FA considered, for both the content and the tissue/diet ratio. For the liver, dividing the numbers of Table 5 (see below) by those of Table 1 gives a set of percentages that can be interpreted as the percentages of the different FA that are incorporated into liver. For plasma, assuming unit density, the ratio comes in a 1/100 scale since the results in plasma are given in µg/mL whereas those in liver are given in µg/100 mg, but the interpretation is the same.

The significance of the differences in FA content and incorporation ratios was also tested by two-way ANOVA. We used SPSS 11.0 (Erkrath, Germany) in the calculations. As usual, $P < 0.05$ was considered significant.

RESULTS

Food intake and body weight. Guinea pigs were healthy throughout the feeding period except for four animals (6%) from groups PE0/PH2, PE1/PH0, PE1/PH2, and PE2/PH1 that died. No relation was found between the diets and the death of these animals. These deaths were taken into account in all measurements, such as body weight, food intake, and FA concentrations. Treatment means (±SD) of food consumption and body weight are shown in Table 3. Although no relevant effect of phytosterol supplementation was detected, we observed a decrease in food consumption from PE1 to PE2, and an increase in body weight from PE0 to PE1. Overall significance tests on pectin effect gave $P = 0.025$ for food consumption and $P < 0.001$ for body weight.

As shown by the curves of Figure 1, all groups gained weight during the study. The differences in weight increase in successive periods were tested. Whereas the pectin effect was significant ($P < 0.001$) from the second week, as shown in Figure 1, the phytosterol effect was not significant (data not shown). Feed efficiency was significantly higher ($P < 0.001$) in the animals fed with pectin than in those fed without, but the phytosterol effect again was nonsignificant.

Fecal FA. The mean content in feces of the seven FA of Table *2* can be seen in Table 4. Comparing the figures in Table 4 with those in Table 2 reveals that the total FA was lower in feces than in the diet, suggesting that fat was better absorbed than the other components of a guinea pig standard diet. Nevertheless, there was a great variability in absorption across the FA spectrum.

Using the ratio of the mean content in feces divided by the content in diet, for the different components of the FA spectrum, we have a rough assessment of apparent absorption. First, we found an overall ratio of 0.35 for total FA. The ratios of the SFA fraction were clearly related to chain length. Thus, the short-chain SFA (8:0 and 10:0) were almost completely absorbed. For the medium- and long-chain SFA, the ratios ranged from 0.12 in lauric acid to 1.07 in stearic acid, with intermediate values for myristic (0.36) and palmitic (0.74) acids. Palmitic and stearic acids were the only ones for which the fecal content was higher than the diet content. MUFA and PUFA ratios must be evaluated more cautiously, since we were dealing with a diet containing highly SFA. Anyway, the ratios for these three FA as reported in Tables 2 and 4 were similar (around 0.16).

Such a variability in fat absorption calls for a separate analysis of the supplementation effects on the different FA. The effect of phytosterol supplementation on the lauric acid content in feces is illustrated in Figure 2, which is a plot of lauric acid content as a function of the phytosterol level, for different levels of pectin. The curves corresponding to levels PE0 and PE1 are remarkably parallel and can be easily related to the variation of the lauric acid content of the diet. The curve corresponding to level PE2 is clearly different, suggesting that high

TABLE 4
Concentrations of the Main FA in Total Fecal Content

		FA[a] (μg/mg)						
Pectin	Phytosterols	12:0	14:0	16:0	18:0	18:1n-9	18:2n-6	18:3n-3
PE0	PH0	8.19	9.81	10.09	14.87	0.81	0.28	ND
		(0.38)	(0.90)	(1.64)	(3.01)	(0.01)	(0.01)	
PE0	PH1	5.60	6.34	8.13	12.85	1.79	0.49	0.12
		(1.23)	(1.31)	(2.48)	(3.99)	(0.62)	(0.17)	(<0.01)
PE0	PH2	5.81	6.32	8.03	13.85	3.08	0.78	0.25
		(1.33)	(1.36)	(1.93)	(4.29)	(0.61)	(0.14)	(0.01)
PE1	PH0	6.76	8.31	9.07	12.92	0.75	0.23	ND
		(2.59)	(2.52)	(2.24)	(4.45)	(0.14)	(0.01)	
PE1	PH1	4.48	5.45	6.83	10.54	1.49	0.42	0.01
		(0.39)	(0.70)	(1.13)	(2.42)	(0.16)	(0.01)	(<0.01)
PE1	PH2	4.38	5.16	7.25	12.75	2.31	0.59	0.16
		(1.10)	(0.95)	(0.84)	(2.16)	(0.44)	(0.19)	(<0.01)
PE2	PH0	6.53	7.72	7.96	11.01	0.68	0.18	ND
		(1.99)	(1.64)	(0.78)	(1.47)	(0.18)	(0.01)	
PE2	PH1	5.57	6.78	7.39	11.63	1.58	0.41	0.01
		(1.25)	(1.21)	(1.35)	(2.65)	(0.57)	(0.16)	(<0.01)
PE2	PH2	3.97	5.12	7.22	10.98	1.96	0.50	0.14
		(0.75)	(0.46)	(1.15)	(2.02)	(0.41)	(0.01)	(<0.01)

[a]FA content expressed as μg/mg of total fecal content (SD in parenthesis). For abbreviations see Tables 1 and 2.

levels of pectin supplementation modulated the effect of phytosterol supplementation, although the small sample size did not allow us clarify this point. The ANOVA test yielded $P < 0.001$ for the phytosterol effect and indicated no significance for the pectin and the interaction effect. Since part of the phytosterol effect could be attributed to the increase of lauric acid content in the diet, one could choose to test the ratio of apparent absorption mentioned above. The result was then $P = 0.044$, which is still significant.

The results of myristic acid, not plotted, were very similar, giving again $P < 0.001$ for the feces content and $P = 0.017$ for the ratio of apparent absorption. Looking at the variation in these ratios, one could see that the phytosterol supplementation decreased the amount of lauric and myristic acid that was excreted by 20%, thus increasing the apparent absorption.

The effect of phytosterol supplementation on palmitic acid content is illustrated in Figure 3. The comparison of the curves shows again the combined effect of phytosterol and pectin supplementation. Here, the decrease in the second part of the curve that is related to pectin level PE2 contrasts with the increase in the palmitic acid content of the diet (Table 2). The test of significance for the phytosterol effect was $P = 0.014$. Although there was a decrease in the feces/diet ratio, from 0.89 in the control diet to 0.70 in the PH2/PE2 diet, the test for this ratio indicated no significant difference, so the conclusions about the phytosterol effect cannot be so firm as for palmitic acid. The results for stearic acid, not plotted here, presented a similar pattern, although the supplementation effects were not significant.

For oleic acid in feces, we found no effect of the addition of pectin, but a significant positive phytosterol effect ($P < 0.001$) correlated with the variation in the content of this FA in the diet. Nevertheless, when looking at the feces/diet ratio, the effects were opposite, so the combined phytosterol and pectin supplementation produced a decrease of 20% with respect to the control diet (PE0/PH0). The results for linoleic acid were similar, with a decrease in the feces/diet ratio of 30%.

Finally, the results of linolenic acid were somewhat different. For the content in feces, we found the same highly significant positive phytosterol effect but a significant negative pectin effect ($P = 0.021$). For the feces/diet ratio, we found negative effects. The pectin effect, in particular, was almost significant ($P = 0.083$).

FA content in liver. Table 5 is a summary of the FA content in liver (μg/100 mg) for the different treatment groups. The SFA fraction, which dominated the FA profile, was quite stable (54–58%). Second came the PUFA fraction, which was more variable (19–29%), and then the MUFA fraction (14–23%).

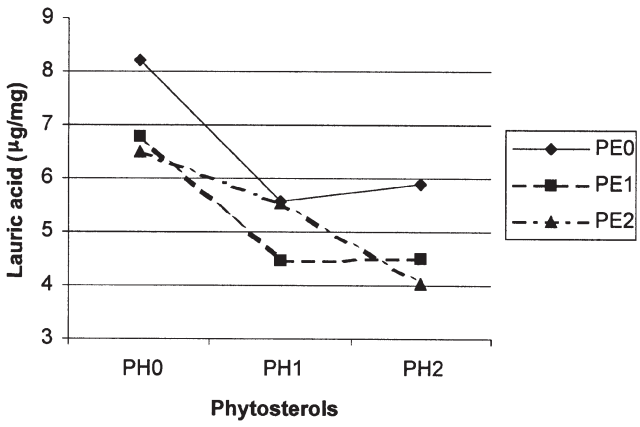

FIG. 2. Concentration (μg/mg) of lauric acid in total feces. PH0, PH1, and PH2, phytosterol levels of 0, 1.37, and 2.45%, respectively; for other abbreviations see Figure 1.

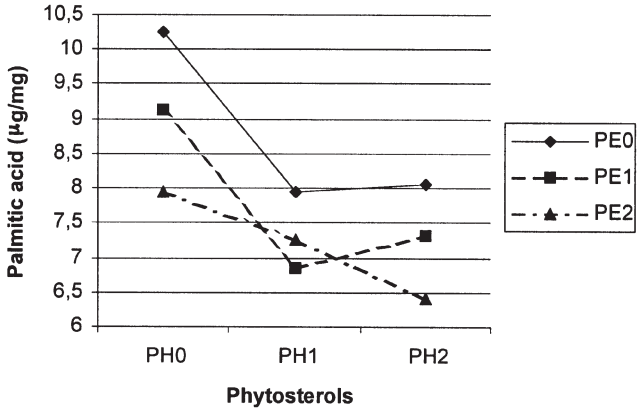

FIG. 3. Concentration (µg/mg) of palmitic acid in total feces. For abbreviations see Figures 1 and 2.

We consistently found, across the different components of the SFA fraction, a nonlinear effect of supplementation, an increase from PH0 to PH1, and a slight decrease from PH1 to PH2. The same effect, but generally weaker, was found for the pectin supplementation. In particular, the highest SFA levels were found when the intermediate doses were combined (PE1/PH1), showing an increase in the SFA liver incorporation ratio from 1.3% in group PE0/PH0 to 2% in group PE1/PH1. The effect of phytosterol on the incorporation ratio was significant ($P < 0.001$), but the pectin effect was not.

Although lauric acid (12:0) was the main component of the FA profile of the diet, it was a minor component in the liver (3–6%), whereas myristic acid (14:0) was more abundant (10–14%). The phytosterol effect on the incorporation ratio was significant in both cases ($P < 0.001$ and $P = 0.002$, respectively), and the pectin effect was not significant. Except for higher doses of pectin (PE2), the phytosterol effect was approximately linear.

Palmitic acid (16:0) was one of the major components of the FA profile in the liver (16–20%). The results for palmitic acid were not significant, although the pectin/phytosterol interaction was almost significant ($P = 0.089$). The content of stearic acid (18:0) was a bit higher (18–23%). Both pectin ($P = 0.002$) and phytosterol ($P = 0.003$) had significant positive effects on this ratio.

The increase in the relative content of oleic acid (18:1n-9) in the diet (2.5–10%) was correlated with the increase in the liver samples (12–16%), but this effect was not significant. The results of linoleic acid (18:2n-6), the last of the major components of the FA profile (12–21%), had a similar pattern, but they were very significant ($P < 0.001$). Finally, for the n-3 PUFA fraction, only linolenic acid was reported, since others were not detected in the diet. The percentage of the n-3 PUFA fraction remained quite stable (11–13%) and was unaffected by changes in these FA in diet.

FA content in plasma. Table 6 is a summary of the FA content in plasma (µg/mL). The percentage of SFA (53–63%) was more variable than in liver, but the levels were similar. Here, palmitic acid was the major component (20–23%), followed by stearic acid (12–14%), myristic acid (10–15%), and lauric acid (4–7%). The percentages of MUFA (19–26%) and PUFA (14–28%) were correlated with the contents of oleic acid and of linoleic and linolenic acids, respectively, in the diet.

The supplementation effects on the total FA content were very clear. As in liver, both pectin and phytosterol supplementation had a nonlinear effect, and the FA content was higher at intermediate doses. As a consequence, the maximal FA content was found when combining intermediate doses (PE1/PH1). For the total FA content, the pectin effect was significant ($P = 0.002$),

TABLE 5
Content of the Main FA in Liver

		FA[a] (µg/mg)						
Pectin	Phytosterols	12:0	14:0	16:0	18:0	18:1n-9	18:2n-6	18:3n-3
PE0	PH0	9.67	29.78	53.77	55.23	36.65	44.39	3.30
		(7.51)	(15.31)	(14.78)	(3.97)	(10.54)	(9.87)	(1.65)
PE0	PH1	9.76	34.20	55.97	56.05	37.69	48.31	4.50
		(3.96)	(11.21)	(13.91)	(4.21)	(9.26)	(7.52)	(1.08)
PE0	PH2	16.70	39.39	51.47	52.27	37.41	57.71	5.78
		(8.71)	(11.76)	(12.82)	(4.78)	(12.19)	(8.09)	(2.45)
PE1	PH0	7.67	23.15	40.26	55.12	30.71	46.04	3.18
		(4.34)	(10.21)	(9.25)	(4.57)	(11.87)	(8.63)	(1.06)
PE1	PH1	16.27	42.23	58.65	59.28	38.37	58.22	5.85
		(10.32)	(17.99)	(20.71)	(6.35)	(11.33)	(8.62)	(2.43)
PE1	PH2	19.96	46.27	56.85	58.93	43.08	67.88	6.35
		(9.20)	(13.21)	(14.50)	(6.18)	(11.92)	(11.09)	(2.08)
PE2	PH0	10.20	33.16	62.98	58.69	48.05	36.56	2.35
		(3.12)	(8.17)	(17.79)	(3.89)	(18.62)	(9.46)	(0.86)
PE2	PH1	17.55	40.53	54.32	56.09	35.11	53.43	5.05
		(14.40)	(30.11)	(32.67)	(6.78)	(18.33)	(8.05)	(3.40)
PE2	PH2	16.81	38.42	46.88	58.70	34.50	61.97	5.33
		(9.75)	(14.46)	(8.72)	(3.26)	(5.81)	(6.39)	(1.51)

[a]FA content expressed as µg/100 mg of liver (SD in parenthesis). For abbreviations see Table 1.

TABLE 6
Content of the Main FA in Plasma

		FA[a] (μg/mL)						
Pectin	Phytosterols	12:0	14:0	16:0	18:0	18:1n-9	18:2n-6	18:3n-3
PE0	PH0	93.16	187.40	316.06	172.33	268.57	195.21	14.36
		(46.79)	(53.17)	(68.12)	(51.68)	(103.83)	(137.09)	(14.94)
PE0	PH1	84.55	206.69	335.52	210.04	275.82	235.70	20.66
		(37.66)	(83.37)	(96.70)	(68.63)	(95.03)	(178.95)	(13.10)
PE0	PH2	78.92	178.01	318.14	189.89	320.84	352.68	19.58
		(30.49)	(32.22)	(96.26)	(46.67)	(150.49)	(109.88)	(14.69)
PE1	PH0	133.33	277.18	357.33	219.49	336.66	315.18	16.68
		(57.83)	(115.90)	(84.65)	(68.73)	(103.44)	(135.30)	(9.83)
PE1	PH1	143.91	266.02	433.06	274.82	389.87	460.62	31.05
		(73.74)	(66.47)	(124.95)	(50.08)	(72.92)	(100.56)	(15.54)
PE1	PH2	65.95	190.34	367.89	234.65	332.49	472.58	35.34
		(15.10)	(47.35)	(124.67)	(84.58)	(104.80)	(136.15)	(28.58)
PE2	PH0	92.59	199.85	344.72	168.04	330.62	171.35	11.03
		(27.46)	(64.58)	(98.62)	(40.04)	(126.80)	(30.03)	(6.71)
PE2	PH1	127.15	212.55	380.97	210.90	344.44	350.63	20.13
		(65.03)	(44.99)	(135.98)	(36.63)	(182.73)	(139.21)	(9.80)
PE2	PH2	70.75	174.45	319.45	211.15	295.22	351.14	23.50
		(20.21)	(25.66)	(55.10)	(54.80)	(37.61)	(72.82)	(8.37)

[a]FA content expressed as μg/mL of plasma (SD in parenthesis). For abbreviations see Table 1.

whereas the phytosterol effect was not, but both effects were significant for the total SFA content ($P = 0.012$ and $P = 0.050$, respectively). Much more significance was found in the plasma/diet ratio, especially for the phytosterol effect ($P < 0.001$). In absolute terms, the type of supplementation used in this study could increase by 50% the total FA content (from 1409 μg/mL in group PE0/PH0 to 2182 μg/mL in group PE1/PH1), without increasing the content in the diet. A similar increase was found in the total SFA content. This pattern was consistently found in all SFA fraction. The effects were stronger for stearic acid and weaker (in decreasing order) for myristic, lauric, and palmitic acids.

The results for oleic acid were again nonsignificant. For linoleic acid, the pectin effect showed the same pattern as in the SFA fraction and was significant ($P = 0.007$). The phytosterol effect was confounded with a diet effect, being positive when the plasma content was used for the comparison, but negative for the plasma/diet ratio. The levels of n-3 PUFA in plasma were very low.

DISCUSSION

Although many research groups in the last 50 years have studied the role of phytosterols and pectin as cholesterol-lowering compounds (7,9,10,20,39–42) few studies have been conducted to determine how these compounds may affect FA metabolism. In the present study, we examined the effect of phytosterol supplementation, added to a diet rich in SFA, on the content of certain FA in plasma and liver and how this effect can be changed when pectin is also supplemented. We decided to add these compounds to a SFA-enriched diet since most of the studies used low-fat products as carriers (43,44). However, to our knowledge, no data are available about the effects of the addition of phytosterols and/or pectin to a Western diet (rich in SFA and cholesterol) (28,29).

In our study, we found a negative effect of pectin supplementation on food consumption, and no effect of phytosterol supplementation. Dietary pectin intake also resulted in a significant body weight increase, which agreed with some results described previously (23), although others (21,45,46) did not find such an effect. We found an increase in feed efficiency at higher doses of pectin, contrary to the results of other studies (45,46). The unexpected increase in feed efficiency and body weight may be due to an early breakdown of this fiber in the gut, although we used high-esterified pectin, which is less fermentable than low-esterified (47,48). This might be explained by taking into account the possible interaction between pectin and the source of added fat, since it has been reported that pectin may cause mucosal proliferation in the lower intestinal tract (49), and this effect can be modulated by the source of fat administered (50,51). Further studies are necessary to clarify the interactions between sources of fat and doses of pectin with different degrees of esterification.

On the other hand, our results are consistent with a previous study (52), in which no effect of different doses of phytosterols on body weight was observed. Despite the high amount of fat in diets enriched with phytosterols (Table 1), our results indicate that slight differences in composition of diets had no effect on growth.

Since the supplementation, whose effects are examined in this paper, was made to a standard guinea pig diet, we deal here with a highly SFA profile. This allows us to emphasize our conclusions on SFA profile but calls for some caution when extending the conclusions about MUFA and PUFA absorption to diets with a completely different FA profile.

Whereas the absorption rates of the MUFA and PUFA examined in this paper look quite uniform, with a feces/diet ratio

of about 0.16, those of SFA are clearly related to the chain length, showing big differences from the short-chain FA (8:0 and 10:0) to long-chain FA. Such a variety suggests that any approach to fat absorption must account for the specificities of the different FA, especially of those related to chain length.

The results for lauric and myristic acid are similar and consistent with the fact that both are medium-chain SFA, so that they should have a similar absorption pathway. The concentration of lauric acid in feces is shown in Figure 2, where the interpretation of the curves corresponding to levels PE0 and PE1 is straightforward: Although the decrease in the first segment can be partly attributed to a decrease in the dietary supply of these FA, the second segment shows that at least for the highest doses, phytosterol supplementation has a negative effect on the fecal content of lauric and myristic acids. When relating the feces content to the diet content, we find a significant positive effect on apparent absorption, suggesting that phytosterols added to a diet enriched with SFA increase the absorption of these FA. Although the conclusions about absorption rates for palmitic acid cannot be so firm, the results point in the same direction, that of a negative effect of phytosterol supplementation. Moreover, the results in plasma and liver agreed with their apparent absorption, in which a significant association was also found between SFA and phytosterol intake.

Unsaturated FA are much less abundant in the diets used in this study, and the results for them are not so clear but point in the same direction, toward a positive effect on FA absorption.

On the other hand, the influence of the dietary fiber intake on SFA is not clear. Whereas some authors (53) reported that the effects of dietary fiber intake might counteract the effects of saturated fat intake and thus reduce CHD events, others (39,54,55) did not find any differences in SFA content in biological samples after fiber ingestion. A recent study (56) reported a liver weight gain in rats fed with a fiber mixture. However, in our study, we did not find a direct effect on FA concentrations after fiber feeding, although pectin modulated the phytosterol effect on FA absorption (results not statistically significant).

The main animal studies (3,57–60) show that dietary phytosterols inhibit the atherosclerotic process, although others (61–65) report an association between increased plasma levels of phytosterols and increased risk of CHD in phytosterolemic and nonphytosterolemic subjects. It remains to be shown whether the high serum plant sterol levels caused by long-term plant sterol ester consumption are harmful. However, taking into account that SFA as lauric, myristic, and palmitic acids have been associated with cardiovascular risk (54,66,67), our results may agree with those who found a potential atherosclerotic effect of plant sterols in the way that they are capable to increase biological SFA concentrations.

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Thia Fatty Acids with the Sulfur Atom in Even or Odd Positions Have Opposite Effects on Fatty Acid Catabolism

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ABSTRACT: As tools for mechanistic studies on lipid metabolism, with the long-term goal of developing a drug for the treatment of lipid disorders, thia FA with the sulfur atom inserted at positions 3–9 from the carboxyl group were fed to male Wistar rats for 1 wk to determine their impact on key parameters in lipid metabolism and hepatic levels of thia FA metabolites. Thia FA with the sulfur atom in even positions decreased hepatic and cardiac mitochondrial β-oxidation and profoundly increased hepatic and cardiac TAG levels. The plasma TAG level was unchanged and the hepatic acyl-CoA oxidase activity increased. In contrast, thia FA with the sulfur atom in odd positions, especially 3-thia FA, tended to increase hepatic and cardiac FA oxidation and acyl-CoA oxidase and carnitine palmitoyltransferase-II activities, and decreased the plasma TAG levels. The effects seem to be related to differences in the catabolic rate of the thia FA. Differences between the two groups of acids were also observed with respect to the regulation of genes involved in FA transport and catabolism. Feeding experiments with 3- and 4-thia FA in combination indicated that the 4-thia FA partly attenuated the effects of the 3-thia FA on mitochondrial FA oxidation and the hepatic TAG level. In summary, the position of the sulfur atom in the alkyl chain, especially whether it is placed in the even or odd position, is crucial for the biological effect of the thia FA.

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Thia FA are modified FA with a sulfur atom inserted in the backbone of a normal FA. In 3-thia FA the sulfur atom is located in the β-position of the alkyl chain, thereby making it non-β-oxidizable. Tetradecylthioacetic acid (TTA), the best-described 3-thia FA, has been extensively tested over the last decade in various animals (1,2), and has recently been subjected to a clinical trial, where it was shown to modify dyslipidemia in HIV-infected humans (3). In many aspects, TTA has properties similar to natural FA, but also has the capacity to profoundly affect lipid metabolism. In test animals TTA has diverse and beneficial effects, including lowering plasma lipids, improving antioxidant status, reducing hyperglycemia, and improving insulin action (reviewed in Ref. 2). These remarkable properties suggest that TTA may have the potential to be used in the prevention and treatment of obesity and obesity-related disorders in humans.

Although the underlying mechanism is not fully understood, TTA is thought to exert its hypolipidemic effect by increasing hepatic FA oxidation, primarily hepatic mitochondrial FA oxidation, and thereby reducing the availability of FA for VLDL synthesis and secretion (4). Research has shown that TTA can enter cells and be activated to its CoA ester (5). TTA is a substrate for carnitine palmitoyltransferase (CPT)-I and CPT-II, and can enter the mitochondria, but the β-oxidation of TTA is blocked since TTA-CoA is not a substrate for acyl-CoA dehydrogenase (6). TTA can be incorporated into several lipid classes but accumulates mostly in the hepatic phospholipids (7). TTA can also be desaturated (8) and elongated (9), and, like a variety of other lipid-like compounds, it transcriptionally activates peroxisome proliferator-activated receptors (PPAR) (10,11). TTA is catabolized by ω-hydroxylation followed by ω-oxidation and oxidation to sulfoxide (12–14). The end products, short sulfoxy dicarboxylic acids, can be detected in the urine of treated animals.

Shifting the position of the sulfur atom toward the methyl end of the alkyl chain renders the thia FA β-oxidizable. The 4-thia FA can undergo one cycle of mitochondrial β-oxidation, but the alkyl-thioacryloyl-CoA formed in the process are poor substrates for both mitochondrial hydratase (15) and for CPT-II (16). Consequently, they accumulate in the mitochondrial matrix, where they inhibit normal FA oxidation (17). 4-Thia FA tend to enlarge the mitochondria but do not promote the proliferation of mitochondria and peroxisomes like the non-β-oxidizable FA (18). In contrast to the effect obtained with the 3-thia FA TTA, administration of 4-thia FA increases the hepatic level of TAG (19), with the formation of numerous fat droplets in the liver cells (18). Thus, intake of 4-thia FA appears to cause a pathological condition, and it may be used as an experimental drug for studies on fatty liver in animal models.

Because of the profound differences between the effects of the 3-thia FA TTA and the 4-thia FA on lipid metabolism, it was of interest to conduct a more extensive study of the consequences of inserting the sulfur atom at different positions in the carbon chain, ranging from the 3- to the 9-position, while keeping the total chain length of the thia FA constant. A survey of

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Abbreviations: ACBP, acyl-CoA binding protein; ACO, acyl-CoA oxidase; CMC, carboxymethyl cellulose; CPT, carnitine palmitoyltransferase; DTA, dodecylthioacetic acid; PPAR, peroxisome proliferator-activated receptor; TTA, tetradecylthioacetic acid.

TABLE 1
Formulas and Trivial Names of the Studied Thia FA[a]

	Abbreviation	Formula	Trivial name
S in odd position	14-Carbon 3-thia	$CH_3(CH_2)_{11}\mathbf{S}CH_2C(O)OH$	Dodecylthioacetic acid (DTA)
	3-Thia	$CH_3(CH_2)_{13}\mathbf{S}CH_2C(O)OH$	Tetradecylthioacetic acid (TTA)
	5-Thia	$CH_3(CH_2)_{11}\mathbf{S}(CH_2)_3C(O)OH$	Dodecylthiobutanoic acid
	7-Thia	$CH_3(CH_2)_9\mathbf{S}(CH_2)_5C(O)OH$	Decylthiohexanoic acid
	9-Thia	$CH_3(CH_2)_7\mathbf{S}(CH_2)_7C(O)OH$	Octylthiooctanoic acid
S in even position	4-Thia	$CH_3(CH_2)_{12}\mathbf{S}(CH_2)_2C(O)OH$	Tridecylthiopropanoic acid
	6-Thia	$CH_3(CH_2)_{10}\mathbf{S}(CH_2)_4C(O)OH$	Undecylthiopentanoic acid
	8-Thia	$CH_3(CH_2)_8\mathbf{S}(CH_2)_6C(O)OH$	Nonylthioheptanoic acid

[a]The thia FA contain 16 carbon atoms with a sulfur atom inserted at different positions in the alkyl chain except for the shorter 3-thia FA DTA, which contains 14 carbon atoms.

the compounds studied in this work is given in Table 1. Of particular interest was how the thia FA would affect gene regulation. Because the 3-thia FA TTA is known to modulate FA transport and catabolism, this issue was one of the main subjects of this study. Additionally, only limited knowledge is available with regard to the metabolic fate of such diverse thia FA, which potentially may have a great impact on their metabolic functions. We therefore determined the level of metabolites of some thia FA. It was previously shown that a similar degree of hypolipidemic effect was not achieved with administration of 3-thia FA with alkyl chains several carbon atoms shorter than that of TTA compared to treatment with TTA (20). However, a 3-thia FA with an alkyl chain two carbons shorter than that of TTA reduced the plasma lipids in the same order as TTA (20). Because this shorter 3-thia FA is the proposed β-oxidation product of the 16-carbon 5-thia FA used in the current experiment, it was of interest to compare the effects of these two thia FA on lipid metabolism.

The long-term goal of studies on thia FA with lipid-lowering effects is to provide a drug with lipid-lowering properties for the treatment of obesity and obesity-related disorders. The screening of thia FA with the sulfur atom in positions 3–9 showed that among thia FA with the sulfur atom in odd positions, the 3-thia FA possessed the most potent biological effects. The thia FA with the sulfur atom in even positions reduced mitochondrial β-oxidation concomitantly with an elevated amount of TAG in the liver and heart. These thia FA obviously are of no clinical significance regarding treatment of obesity-related disorders, but may become important for studies on hepatic lipidosis in animal models. In addition, thia FA with a sulfur atom in odd or even positions are powerful tools in mechanistic studies on the regulation of FA metabolism, and in the present study, we showed that the 4-thia FA partially abolished the biological effects of the 3-thia FA. Thus, the position of the sulfur atom is of major importance for the effects of FA oxidation and lipid levels in the liver and plasma.

MATERIALS AND METHODS

Preparation of thia FA: General procedure. The thia FA (Table 1) were synthesized essentially as previously described (21). Two equivalents of KOH were dissolved in methanol, about 25 mL per g KOH. To the solution, in a stream of argon, was added one equivalent of ω-mercaptocarboxylic acid, $HS(CH_2)$-*n*C(O)OH, and then dropwise one equivalent of the appropriate alkyl bromide, $CH_3(CH_2)m$Br (with $n + m$ being 15 or 13), at room temperature and under constant stirring. Finally, the solution was kept at the reflux temperature for 8–12 h. A sufficient amount of water was then added to dissolve the precipitated KBr, whereupon the pH was adjusted to about 1.5 with HCl. The acidified solution was set aside at about 20°C for 1 to 2 d to allow the thia FA to precipitate and to facilitate the following filtration. The collected acid was washed with water until the filtrate was odorless and was then dried in air. The acid was finally purified by subsequent crystallization from diethyl ether and methanol and dried in an oil pump vacuum. The m.p. was determined with a Perkin-Elmer m.p. apparatus, and all acids had a m.p. range of ±0.5°C. The purity of the compounds was further verified by titration with sodium hydroxide in an inert atmosphere. The solvent was ethanol, and phenolphathalein was used as an indicator. The calculated M.W. of all the FA varied from the theoretical formula weight by 1–5% (data not shown). Mass spectrophotometry verified the molecular structures (data not shown). The analytical methods showed a successful synthesis, with >95% purity of the compounds.

Animals and treatments. Male Wistar rats (Mol:Wist) from Mollegard Breeding Laboratory (Ejby, Denmark), weighing 170–210 g, were housed in pairs in metal wire cages, and both received the same treatment. The rats were kept in a 12-h light/dark cycle at a temperature of 20 ± 3°C and a RH of 65 ± 15% and were acclimatized for at least 1 wk under these conditions prior to the start of the experiments. Thia FA (Table 1) were suspended in 0.5% sodium carboxymethyl cellulose (CMC) and were administered by orogastric intubation once a day for 7 d at doses of 150 (only one group) or 300 mg/d/kg body weight. In one group, the rats were fed 4-thia FA at a dose of 150 mg/d/kg body weight for 4 d and thereafter were fed the 3-thia FA TTA and the 4-thia FA for 7 more days, both at a dose of 150 mg/d/kg body weight. Control rats received palmitic acid suspended in CMC at a dose of 300 mg/d/kg body weight. Each experimental group being fed the thia FA consisted of 4 rats, and the control group consisted of 8 rats. The rats had free access to tap water and standard rat pellet food (rat and mice standard diet No. 1; B&K Universal, Nittedal, Norway). At the end of the feeding period, the animals were fasted for 12 h and

anesthetized subcutaneously with a 1:1 mixture of Hypnorm™ (fentanyl citrate 0.315 mg/mL and fluanisone 10 mg/mL; Janssen Animal Health, Beerse, Belgium) and Dormicum® (midazolam 5 mg/mL; Hoffmann-La Roche, Basel, Switzerland). Cardiac puncture was performed to collect blood samples, and the liver and heart were dissected. Parts of liver and heart were immediately frozen in liquid N_2 while the rest of the liver and heart were chilled on ice for homogenization. The protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals. At the end of the feeding period, no difference in weight gain between the groups could be detected (data not shown).

Homogenization and preparation of subcellular fractions. The liver and heart from the rats were homogenized individually in ice-cold sucrose solution (0.25 M sucrose in 10 mM HEPES buffer, pH 7.4, and 1 mM EDTA) using a Potter–Elvehjem homogenizer. Subcellular fractions were prepared as previously described (22). The procedure was performed at 0–4°C, and the fractions were stored at −80°C. Protein was assayed using the BioRad protein assay kit (BioRad, Heraules, CA) with BSA as standard.

Lipid analysis. TAG in the postnuclear fractions of the liver and heart and in the plasma were measured using the Technicon Axon system (Miles, Tarrytown, NY) with the TAG enzymatic kit (Boehringer Mannheim GmbH, Mannheim, Germany).

To measure the hepatic FA composition, the lipids were extracted from subcellular fractions with 2:1 chloroform/methanol (23). The lipid extracts were added to heneicosanoic acid (21:0) as the internal standard and transesterified in 12% BF_3 in methanol (vol/vol) (24). To remove the neutral sterols and unsaponifiable material, the extracts of FAME were heated in 0.5 mol/L KOH in an ethanol/water solution (9:1, vol/vol). The recovered FA were reesterified using BF_3/methanol. The methyl esters were quantified as previously described (25).

Enzyme assays. The CPT-I and CPT-II activities were measured in the postnuclear fraction using palmitoyl-CoA and [methyl-^{14}C]L-carnitine as substrates (26), and the acyl-CoA oxidase (ACO) activity was measured spectrophotometrically in the postnuclear fraction by a coupled assay using palmitoyl-CoA as substrate (27). Palmitoyl-CoA and palmitoyl-L-carnitine oxidation were measured in the postnuclear fraction as acid-soluble products of [1-^{14}C]palmitoyl-CoA and [1-^{14}C]palmitoyl-L-carnitine, respectively (28).

RNA purification and analysis. Total cellular RNA was isolated by the guanidinium–thiocyanate method (29). Hybridization and preparation of hybridization probes were performed as described earlier (30). The DNA probes were purified fragments of cloned rat genes: CPT-II, 1600 bp *Xho*I/*Xba*I fragment of pBKS-CPT II.4 (31); mitochondrial 2.4-dienoyl-CoA reductase, 632 bp *Pst*I/*Eco*RI insert in pGEM-4Z (32); ACO, 1400 bp *Pst*I fragment of pMJ125 (33); acyl-CoA binding protein (ACBP), 458 bp *Eco*RI fragment of pRL-cACBP –E2b (34). Rat P0 rRNA, 1046 bp *Bam*HI/*Xho*I fragment in pBluescript II SK (z29530, provided by Anders Molven, Bergen, Norway) was used as the control.

Presentation of results. The results are reported as means ± SEM from 4–8 animals in each group. Statistical analyses was performed by one-way ANOVA and Dunnet's *post hoc* test, with the level of statistical significance set at $P < 0.05$. Rats treated with palmitic acid served as the controls.

RESULTS

Thia FA affect the liver weight and hepatic, cardiac, and plasma lipids. It is well documented that peroxisome proliferators can induce hepatomegaly, and previously it was reported that administration of 3-thia FA to rats increased the liver weight (18). Table 2 shows that, in this respect, the 3-thia FA TTA was comparable to the 3-thia FA with two carbon atoms less in the alkyl chain. Thia FA with the sulfur atom in positions 4, 6, or 8 also increased the relative liver weights. 4-Thia FA are known to cause hepatic lipidosis (18), and this study demonstrated that thia FA with the sulfur atom in even positions (4-, 6-, and 8-thia FA) increased the hepatic levels of TAG (Table 3). The 4- and 6-thia FA also increased the TAG level in the heart (Table 3). Hepatic and cardiac TAG levels were unchanged in rats treated with thia FA with the sulfur atom in odd positions, except for the 7-thia FA, which reduced the hepatic TAG level (Table 3). In agreement with previous studies (35,36), the 3-thia FA significantly decreased plasma TAG levels (Table 3). Shifting the position of the sulfur atom further away from the carboxylic end reduced this hypolipidemic effect. Thia FA with the sulfur atom in even positions did not affect the plasma TAG levels (Table 3).

Thia FA change the hepatic and cardiac oxidation of FA. As shown in Figures 1A and 1B, the 3-thia FA increased mitochondrial FA oxidation in the liver and heart. This increase was diminished as the sulfur atom was moved further away from the carboxylic end. In contrast, thia FA with the sulfur atom in an even position decreased mitochondrial FA oxidation in the liver and heart (Figs. 1A, 1B).

Thia FA with the sulfur atom in even or odd positions appeared to enhance the hepatic and cardiac ACO activities, with the 3-thia FA being the far most potent of the thia FA (Fig. 2A). The hepatic

TABLE 2
Effect of Thia FA on the Liver Index with a Dose of 300 mg/d/kg Body Weight for 7 d[a]

	Liver index (% of body weight)
Control (palmitic acid)	3.28 ± 0.07
14-Carbon 3-thia (DTA)	4.42 ± 0.20*
3-Thia (TTA)	4.51 ± 0.27*
5-Thia	3.52 ± 0.14
7-Thia	3.61 ± 0.08
9-Thia	3.59 ± 0.15
4-Thia	3.99 ± 0.12*
6-Thia	4.18 ± 0.06*
8-Thia	4.05 ± 0.09*

[a]The values represent the mean ± SEM for 4 animals in each experimental group and 8 animals in the control group. *$P < 0.05$ compared with the control with palmitic acid. For abbreviations see Table 1.

TABLE 3
Effect of Thia FA on Plasma, Liver, and Heart TAG Levels with a Dose of 300 mg/d/kg Body Weight for 7 d[a]

	TAG		
	Liver (μmol/g tissue)	Heart (μmol/g tissue)	Plasma (mmol/L)
Control	9.8 ± 1.6	2.13 ± 0.43	0.45 ± 0.03
14-Carbon 3-Thia (DTA)	ND	ND	0.11 ± 0.02*
3-Thia (TTA)	8.9 ± 0.5	1.43 ± 0.12	0.19 ± 0.03*
5-Thia	ND	ND	0.24 ± 0.05*
7-Thia	5.6 ± 1.0*	2.01 ± 0.17	0.38 ± 0.03
9-Thia	9.4 ± 0.6	2.42 ± 0.51	0.50 ± 0.09
4-Thia	95.4 ± 7.4*	6.57 ± 0.76*	0.37 ± 0.04
6-Thia	119.8 ± 6.8*	9.85 ± 2.08*	0.59 ± 0.14
8-Thia	78.4 ± 11.4*	3.25 ± 0.80	0.48 ± 0.18

[a]Measurements were taken in plasma or the postnuclear fractions of liver or heart tissues. The values represent the mean ± SEM for 4 animals in each experimental group and 4 to 8 animals in the control group. *$P < 0.05$ compared with the control (palmitic acid). ND, not determined; for other abbreviations see Table 1.

CPT-I and CPT-II activities were increased only in rats fed the 3-thia FA, whereas the cardiac activities of CPT-I and CPT-II remained unchanged (Figs. 2B, 2C).

3- and 5-Thia FA are recovered in hepatic subcellular fractions. In the liver of rats fed the 3-thia FA TTA, this acid was found in equal amounts in the postnuclear, mitochondrial, and peroxisomal fractions (Fig. 3). Likewise, the desaturated product of TTA was found in equal amounts in the different fractions (Fig. 3). In rats fed the shorter (14-carbon) 3-thia FA dodecylthioacetic acid (DTA) or the 5-thia FA, both these acids were recovered in the postnuclear, mitochondrial, and peroxisomal fractions (Fig. 3). This could be explained by the ability of the shorter 3-thia FA to be elongated to a 5-thia FA, and of the 5-thia FA to be chain shortened by one round of β-oxidation. With both diets, there was a higher accumulation of the shorter 3-thia FA DTA than of the 5-thia FA.

The effect of a 3-thia FA is modulated by a 4-thia FA. To further examine the mechanisms behind the opposing effects of 3- and 4-thia FA, an experiment was conducted in which rats were fed a mixture of the 3-thia FA TTA and a 4-thia FA. The rats were first fed the 4-thia FA for 4 d and thereafter with a mixture of the two 3- and 4-thia FA for 7 more days, both at a dose of 150 mg/d/kg. These rats were compared with rats fed 3-thia FA, 4-thia FA, or palmitic acid (control). Figures 4A and 4B show for the first time that the 4-thia FA attenuated the 3-thia FA-mediated increase in mitochondrial FA oxidation. The re-

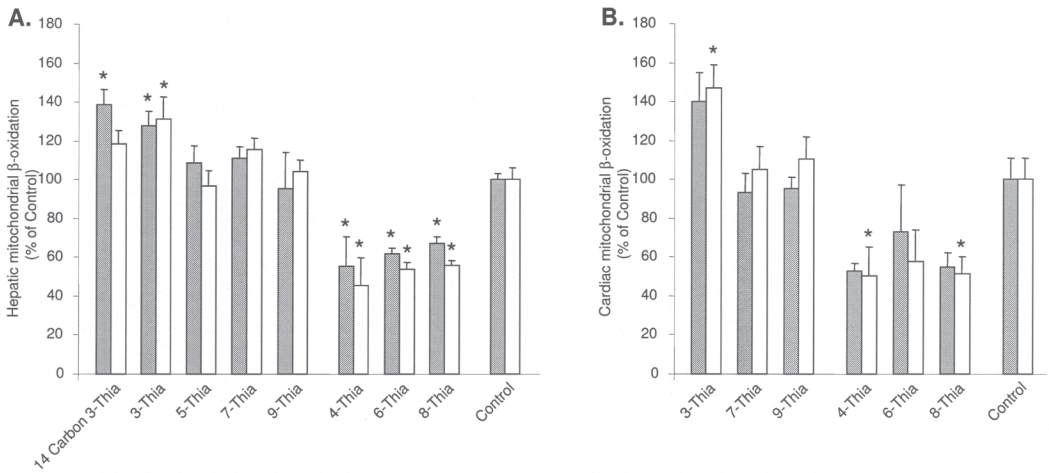

FIG. 1. Mitochondrial FA oxidation in liver (A) and heart (B) in rats fed different thia FA at a dose of 300 mg/d/kg body weight for 7 d. All thia FA (*n*-Thia) contained 16 carbon atoms with a sulfur atom inserted at various positions in the alkyl chain, except for the shorter 3-thia FA, which consisted of 14 carbons and the sulfur atom. The data are presented as the mean ± SEM for 4 animals in each experimental group and 4 to 8 animals in the control group, and as the percentage of control [liver: 1.12 ± 0.04 and 2.02 ± 0.05 nmol/min/mg protein; and heart: 0.57 ± 0.06 and 0.58 ± 0.06 nmol/min/mg protein when palmitoyl-CoA (shaded bars) and palmitoyl-carnitine (open bars) were used as substrates, respectively]. Measurements were performed in the postnuclear fractions of liver or heart tissue. *Significantly different from the control ($P < 0.05$).

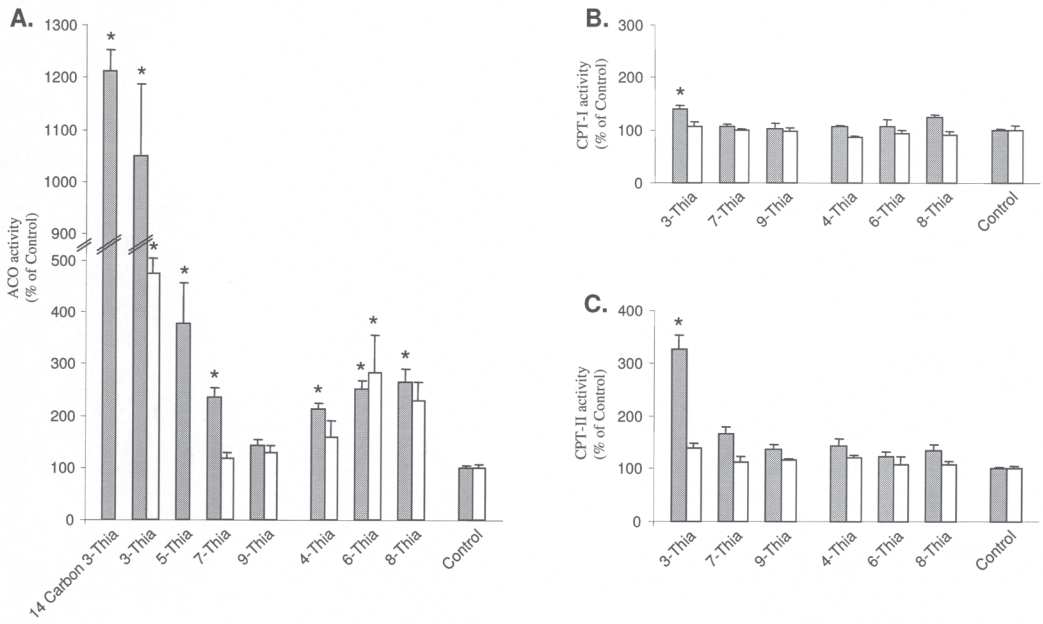

FIG. 2. Activities of acyl-CoA oxidase (ACO) (A), carnitine palmitoyltransferase (CPT)-I (B), and CPT-II (C) in the liver and heart of rats fed different thia FA at a dose of 300 mg/d/kg body weight for 7 d. Nomenclature of the thia FA is as in Figure 1. The data are presented as the mean ± SEM for 4 animals in each experimental group and 4 to 8 animals in the control group and as the percentage of control [ACO: 16.6 ± 0.6 and 0.81 ± 0.05 nmol/min/mg protein; CPT-I: 2.04 ± 0.05 and 1.73 ± 0.15 nmol/min/mg protein; and CPT-II: 10.6 ± 0.3 and 1.00 ± 0.13 nmol/min/mg protein in the liver (shaded bars) and heart (open bars), respectively]. Measurements were performed in the postnuclear fractions. *Significantly different from the control ($P < 0.05$).

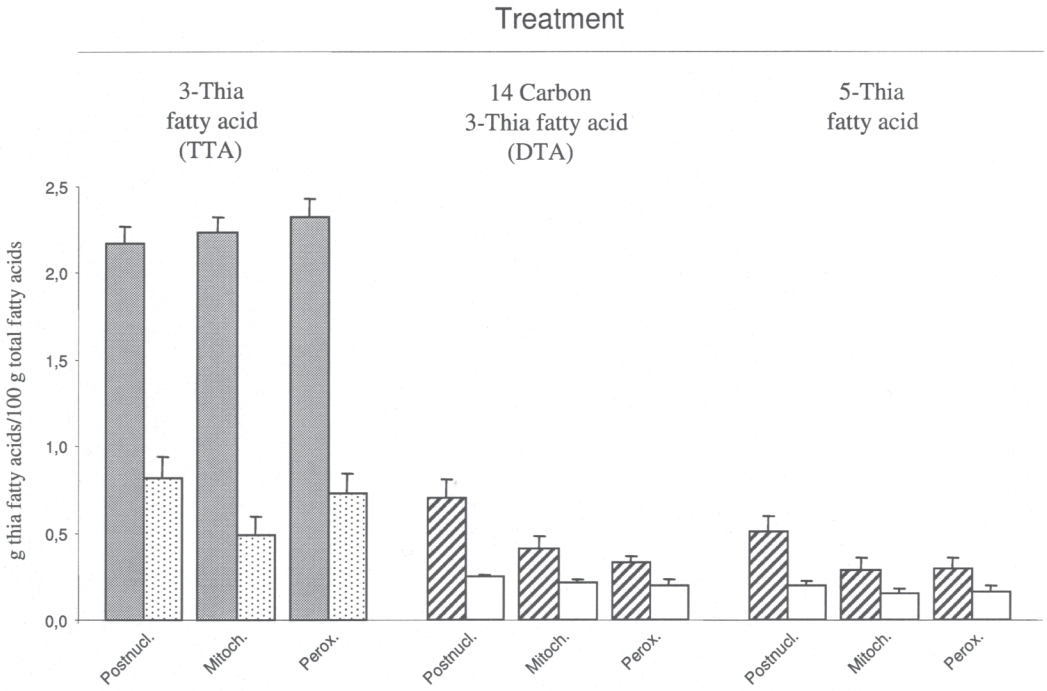

FIG. 3. Accumulation of thia FA in subcellular fractions of the liver of rats fed thia FA at a dose of 300 mg/d/kg body weight for 7 d. The thia FA denoted as 3- and 5-thia FA contained 16 carbon atoms, whereas the shorter 3-thia FA contained 14 carbon atoms. Measurements were performed in the postnuclear, mitochondrial, and peroxisomal fractions of the liver tissue, and the results are presented as grams thia FA per 100 g of the total amount of FA found in the liver, as determined by GC. Thia FA recovered: 3-thia FA (tetradecylthioacetic acid, TTA) (shaded bars); monounsaturated 3-thia FA [TTA:1 (n-8)] (dotted bars); 14-carbon 3-thia FA (dodecylthioacetic acid, DTA) (striped bars); 5-thia FA (open bars). The values represent the mean ± SEM for 4 rats in each group.

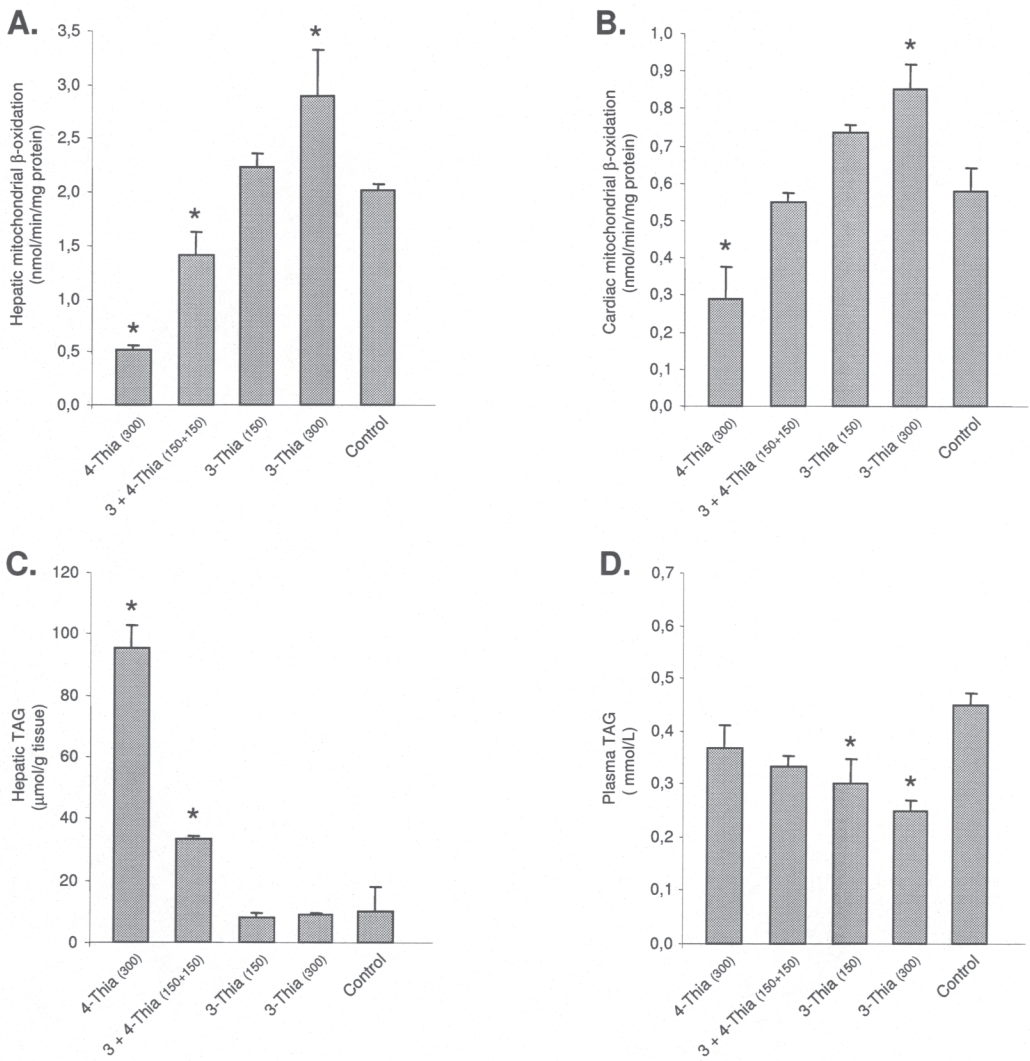

FIG. 4. Effects of 3- and 4-thia FA, and a combination of 3- and 4-thia FA, on (A) hepatic mitochondrial β-oxidation, (B) cardiac mitochondrial β-oxidation, (C) hepatic TAG levels, and (D) plasma TAG levels. (In A and B, palmitoyl-carnitine was used as substrate.) 3-Thia and 4-Thia denote 3- and 4-thia FA, respectively, both with 16 carbon atoms, and the dose in mg/d/kg body weight is given in parentheses. Values are presented as the mean ± SEM. *Significantly different from the control ($P < 0.05$).

duced hepatic FA oxidation was reflected in the elevated hepatic TAG level when feeding a mixture of 3- and 4-thia FA (Fig. 4C). It is noteworthy that the mixture of 3- and 4-thia FA abolished the plasma TAG-lowering effect of the 3-thia FA (Fig. 4D). The 4-thia FA increased the ACO activity and tended to increase the CPT-II activity, although less efficiently than the 3-thia FA (Table 4). Giving rats a combination of 3- and 4-thia FA resulted in higher activities of ACO and CPT-II than did giving the 4-thia FA alone (Table 4). Furthermore, the mixture of 3- and 4-thia FA had an additive effect on the CPT-II activity compared with the lowest dose of the 3-thia FA alone. The gene expression of ACO was increased several fold by the 3-thia FA and tended to be increased by the 4-thia acid, whereas the gene expression of CPT-II was modestly increased by both 3- and 4-thia FA (Table 4). The gene expressions of the mitochondrial 2.4-dienoyl-CoA reductase and ACBP increased with 3-thia FA feeding but were not affected by the 4-thia FA (Table 4). Feeding a combination of 3- and 4-thia FA did not attenuate the 3-thia FA-induced increase in the gene expressions of 2.4-dienoyl-CoA and ACBP (Table 4).

DISCUSSION

Thia FA can generally be viewed as normal FA that at the same time possess some abnormal properties because of the presence of a sulfur atom. This paradoxical statement is based on their normal behavior with respect to transport, activation, esterification, elongation, and desaturation. These compounds apparently act as substrates for most of the lipid-handling enzymes in the cell. In contrast, they are much less susceptible to catab-

TABLE 4
Effect of 3- and 4-Thia FA on Hepatic Enzyme Activities and mRNA Levels[a]

	Control	4-Thia FA	3- and 4-Thia FA	3-Thia FA	3-Thia FA
Dose (mg/d/kg body weight)		300	150 + 150	150	300
Enzyme activities			nmol/min/mg protein		
ACO	16.1 ± 0.6	34.4 ± 1.9*	115 ± 3*	114 ± 16*	201 ± 26*
CPT-II	10.6 ± 0.3	15.1 ± 1.5	23.2 ± 2.4*	16.4 ± 0.9	34.7 ± 2.9*
mRNA levels			relative values		
ACO	1.00 ± 0.10	3.04 ± 0.36	4.51 ± 1.27*	6.95 ± 0.79*	8.77 ± 1.98*
CPT-II	1.00 ± 0.11	2.39 ± 0.23*	2.15 ± 0.55*	2.94 ± 0.39*	2.83 ± 0.38*
2.4-Dienoyl-CoA reductase	1.00 ± 0.25	0.88 ± 0.09	2.61 ± 0.57*	2.64 ± 0.19*	2.72 ± 0.17*
ACBP	1.00 ± 0.04	1.25 ± 0.04	1.72 ± 0.10*	1.67 ± 0.08*	1.68 ± 0.07*

[a]The thia FA contain 16 carbons with sulfur at the 3- or 4-position. Enzyme activities were measured in the postnuclear fractions of liver tissue, and the mRNA levels were measured in total RNA samples extracted from liver tissue. The values represent the mean ± SEM for 4 animals in each group. *$P < 0.05$ compared with the control (palmitic acid). ACO, acyl-CoA oxidase; CPT, carnitine palmitoyltransferase; ACBP, acyl-CoA binding protein.

olization by β-oxidation than the natural FA, and they profoundly affect the oxidation of other FA in the cell. Obviously, the catabolism of thia FA is a decisive factor in determining their biological effects, and this catabolism will be affected by the length of the carbon chain and the position of the sulfur atom. By inserting the sulfur atom in even or odd positions in the carbon chain, two groups of thia FA with distinct features can be identified.

From the data presented in this paper, two important trends can be discerned when the position of the sulfur atom is shifted from the carboxylic end toward the methyl end of the molecule. First, thia FA with the sulfur atom in even or odd positions have opposing effects on the mitochondrial FA oxidation of natural FA. Second, feeding even or odd thia FA increases the ACO activity, a key enzyme involved in the peroxisomal catabolism of FA, with the highest enzyme activity observed after feeding the 3-thia FA. For these acids with the sulfur in odd positions, the ACO activity gradually decreases as the sulfur atom is located farther away from the carboxylic group. This trend is also observed for the plasma and hepatic TAG levels.

3-Thia FA are blocked for β-oxidation, whereas the 5-, 7-, and 9-thia FA will be blocked for further β-oxidation after one, two, or three cycles of oxidation, respectively, until they become shorter 3-thia FA. The hepatic contents of thia FA with the sulfur in odd positions and of their metabolites have recently been analyzed (9), demonstrating that the particular 3-thia FA TTA was recovered in the greatest amount. The 5- and 7-thia FA and their metabolites were recovered in gradually decreasing amounts as the sulfur atom was located closer to the methyl end of the molecule, indicating a more rapid catabolism as the metabolites of shorter carbon chains were formed. Thus, the decrease in biological potency of these thia FA may be related to a diminished effective concentration of the active compound.

In the present experiment, metabolites of the shorter 3-thia FA (DTA) and the 5-thia FA were identified in the nuclear, mitochondrial, and peroxisomal fractions, demonstrating relatively similar levels of metabolites in these fractions (Fig. 3). After feeding the 5-thia FA, both the parent product (0.19 wt%) and the shortened β-oxidized product, 3-thia FA DTA (0.50 wt%), were found in the rat liver (Fig. 3). Presently, it is difficult to assess which of these two compounds is the more active species, or whether they are equally effective. Strangely enough, after feeding the 3-thia FA DTA (equal to the β-oxidized product of the 5-thia FA), the content of this FA in rat liver was no more than 0.70 wt% (Fig. 3) and was comparable to the amount found when feeding the 5-thia FA. However, the biological potency of this compound was stronger than that of the 5-thia FA and was comparable to that of the 3-thia FA TTA (2.17 wt%, Fig. 3). Also in the heart, the shorter 3-thia FA was found in lower amounts than was TTA (20). The reason for this discrepancy is presently not known but may be related to several factors. The biological effect may be determined by an active pool of thia FA or their metabolites in a specific cellular compartment. However, because there were no differences among the contents of the 3-thia FA DTA and of the 5-thia FA in the postnuclear, mitochondrial, and peroxisomal fractions, compartmentalization of the thia FA and their metabolites is probably of minor importance to explain their biological activities. Second, the pharmacokinetics may be different for the thia FA, and especially for the two 3-thia FA. The biological effects of the thia FA may be determined by their *in vivo* concentrations shortly after feeding when these concentrations may be of the same magnitude. Thereafter, the shorter 3-thia FA may have a more rapid catabolism, resulting in a lower recovery of this FA when the animals are killed and analyzed. This may also explain the difference in biological effects after feeding rats the shorter 3-thia FA and the 5-thia FA, even though the hepatic amounts of the shorter 3-thia FA and the 5-thia FA were comparable at the time of analysis and were independent of which thia FA was fed to the rats. The possibility also exists that the shorter 3-thia FA is inherently a more biologically active compound than the other acids.

Thia FA with the sulfur atom in an even position efficiently inhibited mitochondrial FA oxidation in both the liver and heart (Figs. 1, 4). In transfection experiments, the 4-thia FA was found to be a PPARα ligand, although the potency was lower than for the odd-positioned thia FA (Gudbrandsen, O.A., Rost, T., Wergedahl, H., and Berge, R.K., unpublished manuscript). Inherently, the 4-thia FA should, at least to a certain extent, increase FA oxidation. The inhibitory effect of this 4-thia FA can

probably be explained by the formation of a metabolite acting as an inhibitor of mitochondrial FA oxidation (16). This inhibitor, a thioacrylic acid, should theoretically be formed during β-oxidation of all the thia FA with a sulfur atom in an even position, although with gradually shorter carbon chains. Because these acids are equally effective in inhibiting FA oxidation (Fig. 1A), provided the different thioacrylic acids are formed in comparable amounts, the length of the carbon chain does not seem to be decisive for this inhibitory effect.

Thia FA modulated the expression of genes involved in FA transport and catabolism to a variable degree. As PPARα ligands, the 3-thia FA increased the expression of PPARα-regulated genes (Table 4). 4-Thia FA feeding increased, or tended to increase, some of these genes (ACO and CPT-II), whereas others were not affected. When 3- and 4-thia FA were fed together, no additive effect attributable to the 4-thia FA could be detected. ACO activity was affected in a specific manner, determined by the position of the sulfur (Fig. 2A). The increase in ACO activity observed after feeding the odd-positioned thia FA was gradually diminished by moving the sulfur atom toward the methyl end of the molecule. In contrast, the relatively modest effect exerted by feeding the even-positioned thia FA did not seem to be influenced by the position of the sulfur atom.

When interpreting the data obtained from feeding the even-positioned thia FA, one should consider that the hepatic level of TAG is increased (Fig. 4, Table 3) and that the FA composition will be changed, as found in the liver of rats fed 4-thia FA (9). It may be argued that this increase in hepatic lipids may affect the gene expression of specific genes such as ACO and CPT-II in a PPARα-independent manner. The implications are that the metabolic changes exerted by the 4-thia FA and similar acids are due to disturbed lipid metabolism affected by a significantly reduced oxidation of the FA.

The importance of mitochondrial FA oxidation is further emphasized by the results from the experiments in which rats were fed a combination of equal amounts of both 3- and 4-thia FA. This combination resulted in diminished FA oxidation as compared with feeding the 3-thia FA alone. However, 3-thia FA-stimulated oxidation was not completely abolished. Apparently, the inhibitor formed can only partially attenuate the effect of the 3-thia FA, probably due mainly to the increased CPT-II activity. As expected, the rate of FA oxidation observed with the 3- and 4-thia FA, and the combination of these, was reflected in the amount of hepatic TAG, resulting in a reduced hepatic lipidosis when they were combined as compared with feeding the 4-thia FA alone (Figs. 4A–4C). A similar trend was found in the plasma TAG level, although to a lesser extent (Fig. 4D), as the combination of 3- and 4-thia FA decreased the hepatic FA oxidation and abolished the plasma TAG lowering effect of 3-thia FA.

It is reasonable to assume, although difficult to assess directly, that the position of the sulfur atom in the molecule is of minor importance for the direct interaction between the thia FA and regulatory factors such as PPAR. Because of the significant differences in the catabolic rates, this factor is probably more important for the observed differences in metabolic effects. The main regulatory target of these metabolites is mitochondrial FA oxidation, which to a large extent determines how hepatic and plasma lipid metabolism is affected. Thus, 3-thia FA and other acids with the sulfur atom in odd positions increase FA oxidation mainly through a PPAR-mediated mechanism dependent on the actual cellular level of the thia FA present. Thia FA with the sulfur atoms in even positions appear to have metabolic effects governed mainly by significantly reduced FA oxidation in the liver, with a concomitant accumulation of hepatic lipids.

In summary, the results from this study demonstrate the importance of the position of the sulfur atom in the carbon chain when modifying a FA. This position appears to have consequences for the catabolism of the modified FA, which in turn influence interactions with regulatory factors and gene regulation. Among the thia FA with the sulfur atom in odd positions, the 3-thia FA possessed the most potent biological effects, and in the future they may be candidates for use as lipid-lowering drugs for the treatment of obesity and obesity-related disorders. In fact, the 3-thia FA TTA has already been used in a clinical study to treat dyslipidemia in HIV-infected humans on antiretroviral therapy (3). The thia FA with the sulfur atom in even positions reduced the mitochondrial β-oxidation concomitant with an elevated amount of TAG in the liver and heart, and in rats fed the combination of 3- and 4-thia FA, the 4-thia FA partially abolished the biological effects of the 3-thia FA.

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Conjugated Linoleic Acid Reduces Hepatic Steatosis, Improves Liver Function, and Favorably Modifies Lipid Metabolism in Obese Insulin-Resistant Rats

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ABSTRACT: CLA has been shown to induce or suppress excess liver lipid accumulation in various animal models. Interestingly, the state of insulin resistance may be an important modulator of this effect. The objective of the current study was to determine how feeding a dietary CLA mixture would affect liver lipid accumulation in insulin-resistant/obese and lean rats in relation to liver function, lipidemia, liver TAG and phospholipid FA composition, and expression of hepatic markers of FA transport, oxidation, and synthesis. Six-week-old *fa/fa* and lean Zucker rats (n = 20/genotype) were fed either a 1.5% CLA mixture or a control diet for 8 wk. CLA supplementation reduced liver lipid concentration of *fa/fa* rats by 62% in concurrence with improved liver function (lower serum alanine aminotransferase and alkaline phosphatase) and favorable modification of the serum lipoprotein profile (reduced VLDL and LDL and elevated HDL) compared with control-fed *fa/fa* rats. The *fa/fa* genotype had two-thirds the amount of CLA (as % total FA) incorporated into liver TAG and phospholipids compared with the lean genotype. In both genotypes, CLA altered the hepatic FA profile (TAG greater than phospholipids) and these changes were explained by a desaturase enzyme index. Liver-FA-binding protein and acyl CoA oxidase, markers of FA transport and oxidation, respectively, were expressed at higher levels in CLA-fed *fa/fa* rats. In summary, these results illustrate a strong relationship between the state of insulin resistance and liver lipid metabolism and suggest that CLA acts to favorably modify lipid metabolism in *fa/fa* Zucker rats.

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Nonalcoholic fatty liver disease (NAFLD) is a condition that begins with simple hepatic steatosis and may then progress to nonalcoholic steatohepatitis, cirrhosis, and end-stage liver disease (1,2). Hepatic steatosis is defined as excess accumulation of lipid, specifically TAG, in the liver, which may lead to degenerative changes in liver cells. Often, the first clinical signs of hepatic steatosis are abnormal liver function tests. Although, the exact etiology of NAFLD is not known, it is associated with insulin resistance. Consequently, it has been proposed that hepatic steatosis be included as another feature of the metabolic syndrome (1,3). Disturbingly, obesity, insulin resistance, and hepatic steatosis are occurring in children and adolescents at significant rates (4,5). Thus, the investigation of nutritional factors, such as CLA, that may prevent or ameliorate these conditions becomes increasingly relevant.

CLA is a term that describes positional (carbon 7–12) and geometric (*cis-cis*, *cis-trans*, *trans-cis*, and *trans-trans*) isomers of octaecadienoic acid (18:2) (6). CLA induces hepatic steatosis in normal-weight mice (7,8) but ameliorates hepatic steatosis in insulin-resistant obese rats (9–12). These differing outcomes may be due to various factors including species-specific and age-related responses, varying levels and duration of CLA supplementation, and the background diet. However, the response to dietary CLA may also be different in normal vs. obese insulin-resistant states as evidenced in human studies. In men with metabolic syndrome and people with type 2 diabetes, CLA supplementation worsens measures of insulin resistance (13–15), whereas one study in healthy people reported that CLA supplementation improved insulin sensitivity (16).

Thus, the objective of the current 8-wk study was to determine the effects of a CLA mixture (1.5%, w/w) on liver lipid accumulation in relation to (i) liver function, (ii) lipidemia, (iii) liver TAG and phospholipid (PL) FA composition, and (iv) markers of hepatic FA transport, oxidation, and synthesis in 6-wk-old *fa/fa* and lean Zucker rats. Supplementation with a 1.5% (w/w) CLA mixture has been previously shown to improve glucose tolerance in hyperglycemic Zucker Diabetic Fatty (ZDF) rats (17). The *fa/fa* rat has a mutation in the leptin receptor and is an appropriate model for studying hepatic steatosis in the presence of obesity (18,19).

MATERIALS AND METHODS

Animals and diets. Following a 5- to 7-d acclimation period, *fa/fa* (fa) and lean (ln) 6-wk-old Zucker rats (n = 20/genotype; Charles River, St. Constant, Québec, Canada) were randomly assigned to receive either the CLA (faCLA and lnCLA groups) or control (faCTL and lnCTL groups) diet for 8 wk. The diet

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Abbreviations: ACC, acetyl CoA carboxylase; ACO, acyl CoA oxidase; ALT, alanine aminotransferase; CTL, control; fa, *fa/fa* Zucker rats; L-FABP, liver-fatty acid binding protein; ln, lean Zucker rats; mHCl, methanolic hydrochloric acid; MUFA, monounsaturated FA; NAFLD, nonalcoholic fatty liver disease; OLETF, Otsuka Long Evans Tokushima Fatty; PPAR, peroxisome proliferators-activated receptor; RT-PCR, reverse transcriptase polymerase chain reaction; PL, phospholipids; SCD1, stearoyl CoA desaturase 1; SFA, saturated FA; SREBP-1, sterol regulatory binding protein-1; ZDF, Zucker Diabetic Fatty.

TABLE 1
Diet Formulations

Ingredients[a] (g/1000 g)	CLA[b]	CTL[c]
Cornstarch[d]	348	348
Maltodextrin	132	132
Sucrose	100	100
Egg white	213	213
Cellulose	50	50
AIN-93M mineral mix (zinc-free)	35	35
Zinc premix[e]	10	10
Potassium phosphate[f]	5.4	5.4
AIN-93-VX vitamin mix	10	10
Biotin premix[g]	10	10
Choline bitartate	2.5	2.5
TBHQ[h]	0.014	0.014
Soybean oil[i]	70	85
CLA oil[j]	15	0

[a]Ingredients purchased from Harlan Teklad (Madison, WI) unless otherwise indicated.
[b]CLA = CLA diet.
[c]CTL = control diet.
[d]Best Foods (Etobicoke, Ontario, Canada).
[e]Zinc premix = 5.775 g/kg zinc carbonate in cornstarch.
[f]Fisher Scientific (Fair Lawn, NJ), provides mineral content equivalent to AIN-93G diet.
[g]Biotin premix = 200 mg/kg biotin in cornstarch since egg white was the protein source.
[h]Fisher Scientific (Fair Lawn, NJ).
[i]Vita Health (Winnipeg, Manitoba, Canada).
[j]Nu-Chek-Prep, Inc. (Elysian, MN).

TABLE 2
FA Composition of Experimental Diets

% Composition[a]	CLA	CTL
16:0	8.9	10.7
18:0	3.7	4.5
18:1n-9	18.6	22.6
18:2n-6	42.1	51.0
18:3n-3	5.6	6.8
CLA *c*9,*t*11	4.5	0.0
CLA *t*8,*c*10	2.4	0.0
CLA *c*11,*t*13	2.8	0.0
CLA *t*10,*c*12	4.6	0.0
Total SFA[b]	13.3	16.1
Total MUFA[b]	19.9	24.1
Total PUFA	63.7	58.4
Total n-9	18.6	22.7
Total n-6	43.0	51.6
Total n-3	5.6	6.8
Ratios		
PUFA/SFA	4.8	3.6
n-6/n-3	7.7	7.6

[a]% composition = % of total FA.
[b]SFA = saturated FA; MUFA = monounsaturated FA.

formulation was based on the AIN-93G diet (Table 1) (20). The FA distributions of the CLA and CTL diets are shown in Table 2. The dry ingredients for the diets were premixed and stored at 4°C. The CLA oil was aliquoted and stored at –20°C. Fresh batches of the diet, containing oil, were prepared weekly and stored at –20°C until used. Rats were given new feed cups with fresh feed three times per week. Feed consumption (corrected for spillage) and weekly body weights were recorded.

Tissue collection. With cessation of the study period, rats were fasted overnight and euthanized by CO_2 asphyxiation and cervical dislocation according to the Canadian Council on Animal Care Guidelines (21). Trunk blood was collected and immediately placed on ice until centrifuged to separate the serum fraction, which was stored at –80°C. Dissected organs including liver were weighed, immediately frozen in liquid nitrogen, and subsequently stored at –80°C.

Liver lipid concentration. Total liver lipid was separated by chloroform/methanol extraction (22), and total lipid was calculated by weight.

Fasting serum biochemistry. Enzymatic kits were used to quantify serum FFA (#1383175; Roche Diagnostics, Mannheim, Germany), TAG (#DCL 210–75; BioPacific, North Vancouver, Canada), cholesterol (#DCL225–26; BioPacific), alanine aminotransferase (ALT) (#318–10; BioPacific), and alkaline phosphatase (#245; Sigma, St. Louis, MO). Relative serum lipoprotein (VLDL, LDL, HDL) levels were determined using nondenaturing PAGE (23).

FA analysis. Lipids were extracted using a modified Bligh and Dyer extraction procedure (24). Briefly, 0.50 g of liver was added to 10 mL of chloroform/methanol (2:1, vol/vol) with 0.01% BHT (Sigma-Aldrich, Oakville, Ontario, Canada) and extracted and separated by TLC as previously described (25). All solvents were from Fisher Scientific (Nepean, Ontario, Canada).

The two lipid classes were methylated separately using a sodium methoxide ($NaOCH_3$)-based procedure (15 min at 50°C) to ensure that isomerization of double bonds in the conjugated PUFA did not occur (26). Analyzing the PL fraction required the use of two methylating agents since there is no one methylating agent that is appropriate for analyzing both CLA and the FA found in sphingomyelin [i.e., sphingomyelin FA are not methylated by $NaOCH_3$ and CLA is isomerized by acid-based methylating agents (27)]. Thus, a duplicate PL fraction was also methylated with methanolic hydrochloric acid (mHCl) for 2 h at 80°C. The results from the two methylation procedures were combined based on quantification by the internal standard 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (Avanti, Alabaster, AL) added to duplicate samples in equal amounts. For GC, $NaOCH_3$-methylated samples were separated on a Chrompack CP-select CB column (100 m × 0.25 mm diameter and 0.25 μm film thickness; Varian Canada Inc., Mississauga, Ontario) using a Varian CP-3800 GC with FID. The column was operated at 45°C for 4 min, the temperature was raised to 175°C at 13°C/min, held for 27 min, raised again to 190°C at 1°C/min, held for 38 min, raised again to 215°C at 4°C/min, held for 10 min, and finally raised to 240°C at 4°C/min and held for 5 min. Total run time was 121.5 min, and samples were run with a 10:1 split ratio. The mHCl-methylated samples were separated on a DB225MS column (30 m × 0.25 mm diameter and 0.25 μm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario) using a Varian 3400 GC with FID. The temperature program was 70°C for 1 min,

the temperature was raised to 180°C at 20°C/min, held for 8.5 min, raised to 220°C at 3°C/min, held for 15 min, and finally raised to 240°C at 20°C/min and held for 10.5 min. Total run time was 55 min, and samples were run with a 20:1 split ratio. Individual FA results are expressed as the percentage of total FA. The Δ^9 desaturase index [(16:1n-7 + 18:1-n9)/(16:0 + 18:0)] was calculated based on the FA results.

Reverse transcription-polymerase chain reaction (RT-PCR) for analysis of mRNA. Markers of hepatic FA transport, oxidation, and synthesis [liver-FABP (L-FABP), acetyl CoA oxidase (ACO), and acetyl CoA carboxylase (ACC) mRNA levels, respectively] were assessed by RT-PCR using previously published procedures (28). Briefly, total RNA was isolated using TRIzol (Invitrogen Corp., Burlington, Ontario, Canada). The RNA was resuspended in RNase-free water, and concentration determined by spectrophotometric absorbance at 260 nm. Reverse transcription of 1 μg RNA was conducted (after removal of possible genomic DNA contamination with DNase I) according to the protocol (25 cycles of amplification and 62°C annealing temperature) recommended for the Access RT-PCR system (Promega, Madison, WI). The sequences for the PCR primers (Invitrogen Life Technologies, Burlington, Ontario, Canada) were ACO (sense) 5′-TTCGAGGCTTGGAAACCACT-3′, ACO (antisense) 5′-CTGGGCGTATTTCATCAGCA-3′, ACC (sense) 5′-AGGATCTTAAGGCCAACGCA-3′, ACC (antisense) 5′-TTGGGATCTGCAATGTCTGG-3′, L-FABP (sense) 5′-CGGCAAGTACCAAGTGCAGA-3′, L-FABP (antisense) 5′-CTGCTTGACCTTTTCCCCAG-3′, and the housekeeping gene, L32 ribosomal protein (sense) 5′-TAAGCGAAACTGGCGGAAAC-3′ and L32 ribosomal protein (antisense) 5′-GCTCGTCTTTCTACGATGGCTT-3′. The Gene Bank accession numbers were M13501, AB004329, NM022193, and M13501 for L-FABP, ACO, ACC, and L32, respectively. Vistra green (Amersham, Baie d'Urfe, Québec, Canada) was used to visualize RT-PCR products on 2% agarose gels by electophoresis. Relative band intensity was quantified by scanning the gel (Storm Fluorimager, Amersham) and results are expressed as arbitrary units.

Statistical analysis. Two-way ANOVA was used to determine significant main effects (genotype, lipid, and genotype × lipid interaction), and Duncan's multiple range test was used for means testing (SAS 6.04; SAS Institute, Cary, NC). Data are presented as mean ± SEM unless otherwise indicated. For mRNA and lipoprotein data, differences were assessed by contrasts of preplanned comparisons. The significance level was $P < 0.05$.

RESULTS

Liver lipid content and function. The *fa/fa* rats weighed 58% more than lean rats (567 ± 10 vs. 358 ± 6 g) and consumed 43% more feed than lean rats (1539 ± 27 vs. 1078 ± 22 g), but there were no differences due to dietary treatment (data not shown). The *fa/fa* rats had greater liver weight, relative liver weight, and liver lipid concentration compared with lean rats (Table 3, Fig. 1). Dietary CLA reduced liver weight by 40%, relative liver weight by 37%, and liver lipid concentration by 62% in the faCLA rats compared faCTL rats. In fact, the relative liver weight of faCLA rats was equivalent to that of the lean rats (Table 3). This was paralleled by 54 and 64% reductions in fasting serum ALT and alkaline phosphatase (markers of liver function), respectively, in faCLA rats compared with faCTL rats. Although the faCLA rats still had a twofold higher serum ALT than the lean controls, serum alkaline phosphatase was not different between faCLA, lnCTL, and lnCLA rats (Fig. 2).

Lipidemia. The *fa/fa* rats had higher fasting serum FFA (0.62 ± 0.03 vs. 0.27 ± 0.02 mmol/L), TAG (16.55 ± 0.94 vs. 3.90 ± 0.31 mmol/L), and cholesterol (7.80 ± 0.56 vs. 3.16 ± 0.12 mmol/L) compared with the lean rats. There was no effect of CLA on fasting serum FFA or TAG; however, fasting serum cholesterol was 37% lower in faCLA rats compared with faCTL rats (Table 3). The *fa/fa* rats had elevated fasting serum VLDL and LDL and reduced HDL compared with the lean rats. Dietary CLA reduced fasting serum VLDL by 60% and LDL by 41% in faCLA rats compared with faCTL rats (Fig. 3). Fasting serum HDL in faCLA rats was elevated to a level comparable with lean rats.

Liver FA composition. (i) CLA isomers. The faCLA rats had only two-thirds the CLA isomers present in liver TAG and PL compared with lnCLA rats, as a percentage of total FA (Fig. 4).

TABLE 3
Characteristics of *fa/fa* and Lean Zucker Rats Fed 0 or 1.5% CLA for 8 wk[a]

					Pr > *F*		
	faCLA	faCTL	lnCLA	lnCTL	Geno	Lipid	Geno × lipid
Liver weight							
Liver (g)	17.9 ± 0.6[b]	29.7 ± 0.9[a]	11.1 ± 0.3[c]	10.0 ± 0.9[c]	<0.0001	<0.01	<0.0001
Liver (g/100 g bwt)	3.2 ± 0.1[b]	5.1 ± 0.1[a]	3.1 ± 0.1[b]	2.8 ± 0.2[b]	<0.0001	<0.0001	<0.0001
Lipidemia							
FFA (mmol/L)	0.68 ± 0.05	0.56 ± 0.03	0.26 ± 0.02	0.28 ± 0.03	<0.0001	NS	NS
TAG (mmol/L)	18.03 ± 1.58	15.07 ± 0.70	3.76 ± 0.48	4.05 ± 0.43	<0.0001	NS	NS
Cholesterol (mmol/L)	6.01 ± 0.47[b]	9.59 ± 0.63[a]	2.94 ± 0.15[c]	3.38 ± 0.16[c]	<0.0001	<0.0001	<0.01

[a]Means ± SEM for $n = 10$/group; main effects from ANOVA: Geno = genotype (*fa/fa* vs. lean rats), Lipid (0 vs. 1.5% CLA), and Geno × lipid = lipid × genotype interaction; means with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple range test; faCLA = *fa/fa* rats fed 1.5% CLA, faCTL = *fa/fa* rats fed 0% CLA, lnCLA = lean rats fed 1.5% CLA, lnCTL = lean rats fed 0% CLA; bwt = body weight; NS = not significantly different.

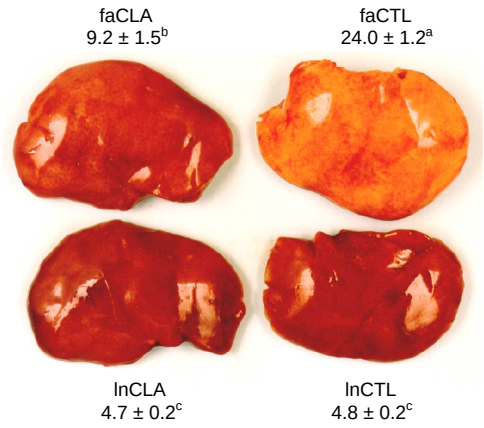

FIG. 1. Hepatic steatosis and liver lipid concentration (%). The pale color of faCTL liver portrays hepatic steatosis, which was confirmed by analysis of liver lipid concentration; values are means ± SEM for $n = 10$ rats/group; means with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple range test; faCLA = *fa/fa* rats fed 1.5% CLA, faCTL = *fa/fa* rats fed 0% CLA, lnCLA = lean rats fed 1.5% CLA, lnCTL = lean rats fed 0% CLA.

The faCLA rats had less *c*9,*t*11/*t*8,*c*10 CLA in liver TAG, and less *c*11,*t*13 CLA in liver PL compared with lnCLA rats. The dominant isomers in liver TAG were *c*9,*t*11/*t*8,*c*10, whereas in liver PL the isomers reported were equally distributed in the faCLA rats. In contrast, lnCLA rats had a greater proportion of *c*11,*t*13 compared with the other isomers.

(ii) Liver TAG. The *fa/fa* rats had higher total saturated FA (SFA) (40.7 ± 0.6 vs. 27.9 ± 1.0%) and monounsaturated FA (MUFA) (38.6 ± 2.7 vs. 24.5 ± 1.2%) and lower PUFA (18.0 ± 2.5 vs. 44.1 ± 2.1%) than lean rats. CLA feeding resulted in a lower proportion of MUFA and a higher proportion of PUFA in liver TAG of *fa/fa* rats (Table 4). The Δ^9 desaturase index was reduced in faCLA rats compared with faCTL rats. The PUFA/SFA ratio was lower in *fa/fa* rats compared with lean rats (0.45 ± 0.07 vs. 1.61 ± 0.12) and unchanged by dietary CLA treatment. The faCLA rats had a lower proportion of n-9 (18:1n-9) and a higher proportion of n-6 (18:2n-6, 20:4n-6) and n-3 (18:3n-3, 20:5n-3, 22:5n-3, 22:6n-3) FA in liver TAG compared with faCTL, resulting in a pattern more similar to lean rats. Interestingly, the n-6/n-3 ratio was lowest in faCLA compared with faCTL and lean rats.

(iii) Liver PL. The *fa/fa* rats had higher SFA (45.1 ± 0.4 vs. 42.7 ± 0.4%) and MUFA (6.18 ± 0.43 vs. 5.29 ± 0.19%) and lower PUFA (45.9 ± 1.3 vs. 49.0 ± 0.7%) and PUFA/SFA ratio (1.02 ± 0.04 vs. 1.15 ± 0.03) compared with lean rats. CLA-fed rats had a lower proportion of MUFA (5.05 ± 0.21 vs. 6.43 ± 0.34%) and a lower Δ^9 desaturase index compared with CTL-fed rats (Table 5). The faCLA rats had a lower Δ^9 desaturase index compared with faCTL and lean rats. The faCLA and lean rats had similar proportions of n-9 (18:1n-9) FA, which were lower than the faCTL group. The *fa/fa* rats had a smaller proportion of total n-6 (due to 18:2n-6) FA than lean rats. CLA-fed rats had more 18:2n-6 than CTL-fed rats, and faCLA rats had more 20:3n-6 than faCTL rats. Arachidonic acid (20:4n-6) was not affected by genotype or diet. Total n-3 FA were not changed, but CLA-fed rats had a greater proportion of 22:5n-3 than CTL-fed rats.

Hepatic gene expression. The faCLA rats had higher L-FABP mRNA levels and a trend ($P = 0.0589$) toward greater ACO mRNA levels compared with faCTL rats (Fig. 5). There was no difference in ACC mRNA levels within the *fa/fa* genotype. In lean rats, there were no differences in L-FABP, ACO, and ACC mRNA levels.

DISCUSSION

CLA isomers were incorporated into liver and affected liver lipid metabolism. Although the effects of dietary CLA on tissue CLA incorporation and FA composition have been investigated in rodent models, there is a lack of information comparing the response in normal vs. obese insulin-resistant states. In the present study, the *fa/fa* rats had just two-thirds the percentage of CLA in liver TAG and PL compared with lean rats (Fig. 4), suggesting that CLA metabolism (e.g., oxidation, storage) is altered in the obese insulin-resistant state. It has been shown by others that *t*10,*c*12 CLA is metabolized to a greater extent

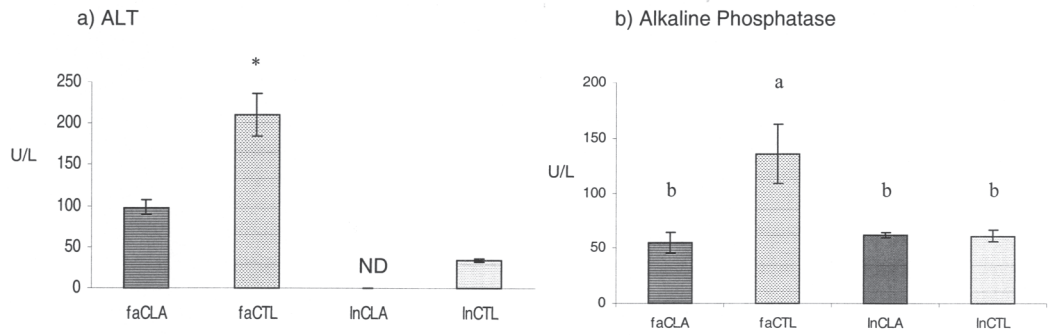

FIG. 2. Fasting serum (a) alanine aminotransferase (ALT) and (b) alkaline phosphatase. Results expressed as means ± SEM for $n = 10$ rats/group; * denotes faCTL different from faCLA and lnCTL ($P < 0.05$); means with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple range test. ND = not determined; U/L = units per liter; for other abbreviations see Figure 1.

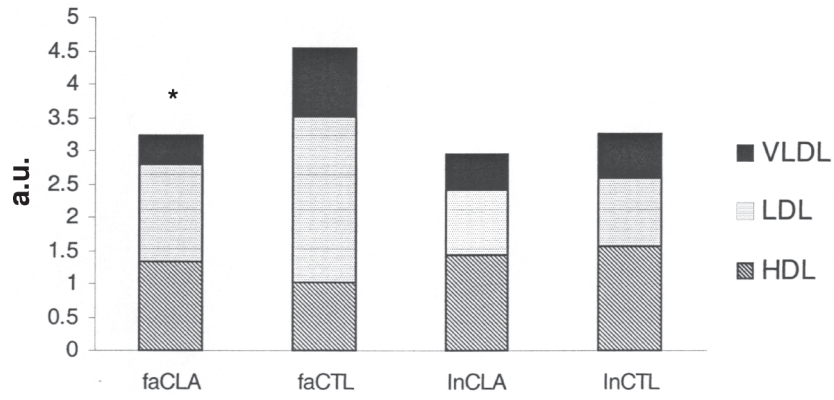

FIG. 3. Fasting serum lipoproteins. Results expressed as means for arbitrary units for lipoprotein bands for $n = 10$ rats/group except $n = 9$ for lnCLA; * denotes faCLA different from faCTL for VLDL, LDL, and HDL using pairwise comparisons ($P \leq 0.05$); a.u., arbitrary units; for other abbreviations see Figure 1.

than other isomers (30), and both the *fa/fa* and lean genotypes had more *c*9,*t*11/t8,*c*10 CLA than *t*10,*c*12 CLA in liver TAG (Table 4). Interestingly, the lnCLA rats had proportionally more *c*11,*t*13 in liver PL (Fig. 4), but, given the relatively small percentage of CLA found in liver PL, the biological significance of this remains to be determined.

The present study demonstrated that a dietary CLA mixture ameliorates hepatic steatosis (62% less total lipid; Fig. 1) in *fa/fa* Zucker rats, consistent with other studies using rat models of obesity and insulin resistance (9–12). Although the progression of NAFLD was not monitored by histology in this study, fasting serum ALT and alkaline phosphatase were lower (54 and 64%, respectively) in *fa/fa* CLA-fed rats compared with *fa/fa* CTL-fed rats (Fig. 2), illustrating the influence of excessive fat accumulation on declining liver function. In people with the metabolic syndrome, interventions that improve insulin sensitivity (e.g., weight loss, oral antihyperglycemic agents) are potential therapies for the treatment of hepatic steatosis and may improve liver function tests (31). One possible reason for the species-specific effect of CLA on insulin resistance may be differences in liver lipid content mediated by CLA. For example, CLA supplementation in mice results in hepatic steatosis and worsening of insulin resistance (7), whereas in the *fa/fa* Zucker rat, reduction of hepatic steatosis by CLA appears to be associated with improved insulin sensitivity (12,32). To date, the effects of CLA supplementation on hepatic steatosis in humans have not been reported.

Circulating lipids portray a balance between liver lipid processing and peripheral lipid storage and release. Supplementation with a CLA mixture did not alter fasting serum TAG or FFA in male (present study; Table 3) or female (32) *fa/fa* Zucker rats. However, circulating TAG and FFA were reduced by CLA supplementation in ZDF rats (CLA mixture) (33), OLETF (Otsuka Long Evans Tokushima Fatty) rats (CLA mixture) (9), normal mice (*c*9,*t*10 or *t*10,*c*12) (34), and ob/ob mice (*c*9,*t*10 but not *t*10,*c*12) (35). It is noteworthy that in the present study, the circulating FFA and TAG levels in the *fa/fa* CLA-fed rats did not change despite the central role of the liver in lipid processing and the dramatic reduction in liver lipid concentration.

In the present study, CLA supplementation reduced fasting serum total cholesterol (Table 3) and VLDL and LDL fractions

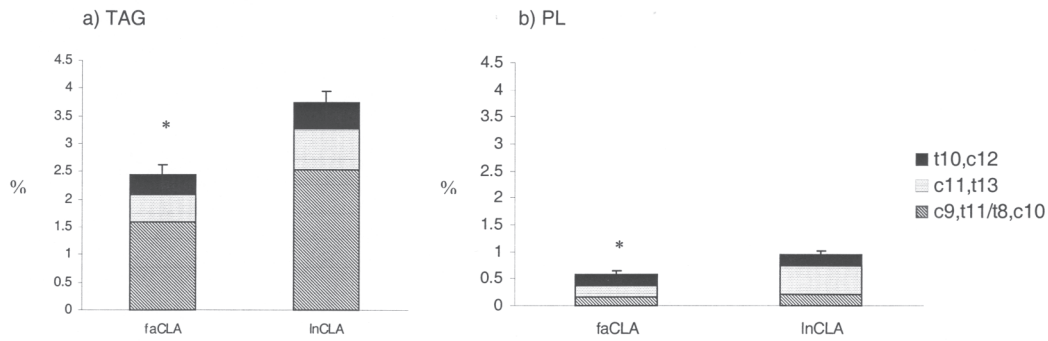

FIG. 4. CLA isomers in (a) TAG and (b) phospholipids (PL) from liver. Results (% of total FA) expressed as means for separate CLA isomers and means ± SEM for total CLA for $n = 5$ rats/group; * denotes faCLA different from lnCLA ($P < 0.05$) for total CLA (TAG and PL), *c*9,*t*11 (TAG) and *c*11,*t*13 (PL); *c*9,*t*11/*t*8,*c*10 = sum of *c*9,*t*11 and *t*8,*c*10 because these isomers coelute (29); for other abbreviations see Figure 1.

TABLE 4
Liver TAG FA Composition of *fa/fa* and Lean Zucker Rats Fed 0 or 1.5% CLA for 8 wk[a]

					Pr > *F*		
	faCLA	faCTL	lnCLA	lnCTL	Geno	Lipid	Geno × lipid
% Composition							
14:0	2.09 ± 0.13	1.64 ± 0.03	1.05 ± 0.07	0.87 ± 0.13	<0.0001	<0.01	NS
16:0	34.49 ± 0.70[a]	36.72 ± 0.47[a]	26.61 ± 0.82[b]	23.26 ± 1.31[c]	<0.0001	NS	<0.01
16:1n-9	0.55 ± 0.04	0.60 ± 0.15	0.23 ± 0.10	0.46 ± 0.12	<0.05	NS	NS
16:1n-7	6.89 ± 0.12[b]	9.75 ± 0.42[a]	2.69 ± 0.32[c]	2.59 ± 0.76[c]	<0.0001	<0.01	<0.01
18:0	3.37 ± 0.33[a]	2.36 ± 0.17[b]	1.64 ± 0.06[c]	1.56 ± 0.20[c]	<0.0001	<0.05	<0.05
18:1n-9	20.61 ± 0.88[b]	32.14 ± 0.40[a]	17.60 ± 0.83[c]	19.28 ± 1.32[b,c]	<0.0001	<0.0001	<0.0001
18:1n-7	2.07 ± 0.08[b]	3.58 ± 0.36[a]	1.77 ± 0.13[b]	2.12 ± 0.20[b]	<0.01	<0.01	<0.01
18:2n-6	14.38 ± 0.91	8.91 ± 0.56	33.27 ± 1.25	33.53 ± 2.57	<0.0001	<0.01	NS
18:3n-6	0.23 ± 0.06	0.26 ± 0.02	0.64 ± 0.03	0.72 ± 0.19	<0.01	NS	NS
18:3n-3	1.77 ± 0.14[b]	0.54 ± 0.06[c]	2.26 ± 0.07[a]	2.19 ± 0.18[a]	<0.0001	<0.0001	<0.01
20:4n-6	2.16 ± 0.37[a]	0.49 ± 0.04[b]	2.37 ± 0.19[a]	3.58 ± 0.68[a]	<0.01	NS	<0.01
20:5n-3	0.50 ± 0.05[a]	0.09 ± 0.01[b]	0.46 ± 0.13[a]	0.64 ± 0.19[a]	<0.05	NS	<0.05
22:5n-3	0.74 ± 0.09[a]	0.15 ± 0.01[b]	0.58 ± 0.08[a]	0.46 ± 0.14[a]	NS	<0.01	<0.05
22:6n-3	2.35 ± 0.81[a]	0.32 ± 0.09[b]	1.61 ± 0.19[a,b]	1.86 ± 0.46[a]	NS	NS	<0.05
Totals							
SFA	40.47 ± 1.10	40.92 ± 0.61	29.47 ± 0.85	26.25 ± 1.35	<0.0001	NS	NS
MUFA	30.75 ± 0.86[b]	46.53 ± 1.06[a]	23.43 ± 1.29[c]	25.63 ± 2.13[c]	<0.0001	<0.0001	<0.01
PUFA	25.12 ± 1.70[b]	10.89 ± 0.74[c]	44.91 ± 1.99[a]	43.19 ± 3.95[a]	<0.0001	<0.01	<0.05
SAT + MUFA + PUFA	96.34 ± 1.36	98.34 ± 0.55	97.81 ± 0.67	95.07 ± 2.15	NS	NS	NS
n-9	21.21 ± 0.87[b]	32.85 ± 0.49[a]	17.84 ± 0.88[c]	19.80 ± 1.29[b,c]	<0.0001	<0.0001	<0.0001
n-6	17.77 ± 1.05[b]	9.79 ± 0.59[c]	37.00 ± 1.51[a]	38.03 ± 3.32[a]	<0.0001	NS	0.0323
n-3	5.37 ± 0.91[a]	1.10 ± 0.16[b]	4.90 ± 0.43[a]	5.15 ± 0.75[a]	<0.05	<0.01	<0.01
Ratios							
MUFA/SFA	0.76 ± 0.03[c]	1.14 ± 0.04[a]	0.79 ± 0.03[c]	0.97 ± 0.04[b]	NS	<0.0001	<0.05
PUFA/SFA	0.63 ± 0.06	0.27 ± 0.02	1.54 ± 0.11	1.68 ± 0.21	<0.0001	NS	NS
n-9/n-6	1.21 ± 0.10[b]	3.41 ± 0.25[a]	0.49 ± 0.04[c]	0.55 ± 0.08[c]	<0.0001	<0.0001	<0.0001
n-6/n-3	3.65 ± 0.52[b]	9.43 ± 1.02[a]	7.70 ± 0.46[a]	7.73 ± 0.75[a]	NS	<0.01	<0.01
Δ^9 DS index[b]	0.73 ± 0.03[c]	1.07 ± 0.03[a]	0.72 ± 0.03[c]	0.88 ± 0.04[b]	0.0050	<0.0001	<0.05

[a]Means ± SEM for $n = 5$ rats/group; main effects from ANOVA: Geno = genotype (*fa/fa* vs. lean rats), Lipid (0 vs. 1.5% CLA), and Geno × lipid = lipid × genotype interaction; means with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple range test; faCLA = *fa/fa* rats fed 1.5% CLA, faCTL = *fa/fa* rats fed 0% CLA, lnCLA = lean rats fed 1.5% CLA, lnCTL = lean rats fed 0% CLA; NS = not significantly different. Only FA present at >0.25% composition are reported.
[b]Δ^9 DS index = [(16:1n-7 + 18:1n-9)/(16:0 + 18:0)].

(Fig. 3) in *fa/fa* Zucker rats; the HDL level in *fa/fa* Zucker rats was elevated to the level of lean rats (Fig. 3). Others have reported that serum total cholesterol was reduced in OLETF rats (10) and peroxisome proliferator activated receptor α (PPARα)-null mice fed CLA mixtures (36) and in hamsters fed the *t*10,*c*12 isomer (37). In OLETF rats, dietary CLA reduced hepatic microsomal TAG transfer protein activity, which is involved in lipoprotein packaging (9), and this would contribute to reduced circulating VLDL. In healthy adults, 80:20 *c*9,*t*11/*t*10,*c*12-CLA supplementation lowered fasting plasma VLDL-cholesterol but did not affect HDL- and LDL-cholesterol (38). However, in men with the metabolic syndrome, fasting plasma HDL-cholesterol was unfavorably decreased by supplementation with a CLA mixture or *t*10,*c*12 CLA (13) but not *c*9,*t*11 CLA (14). In insulin-resistant humans, HDL carries much more TAG and is catabolized by endothelial lipase to a much greater extent compared with non-insulin-resistant persons (39).

CLA is a PPARα-ligand, and both L-FABP and ACO contain PPAR response elements (40). In the present study, the mRNA levels of hepatic PPARα did not change (data not shown). However, CLA-fed *fa/fa* rats had higher mRNA levels of PPAR-responsive genes that are markers of hepatic FA transport (L-FABP) and oxidation (ACO, $P = 0.0589$; Fig. 5) concomitant with reduced liver lipid. It is unclear why CLA supplementation did not alter these PPAR-responsive genes in the lean rats. Interestingly, the genes involved in FA oxidation (peroxisomal and mitochondrial) and transport are elevated in the liver of CLA-fed mice and rats, independent of whether CLA induces or ameliorates hepatic steatosis (8,10,31,41–43). Furthermore, up-regulation of genes for FA oxidation can occur independently of PPARα activation, based on studies using mice deficient in both PPARα and stearoyl CoA desaturase 1 (SCD1) (44). All together, the mechanism by which CLA induces or protects against hepatic steatosis in mice and rats, respectively, appears to be independent of PPARα and FA oxidation. Regulation of FA synthesis or elongation may play a greater role.

Sterol regulatory binding protein (SREBP)-1 up-regulates FA synthesis and elongation genes, including ACC and SCD1

TABLE 5
Liver Phospholipid FA Composition of *fa/fa* and Lean Zucker Rats Fed 0 or 1.5% CLA for 8 wk[a]

					Pr > *F*		
	faCLA	faCTL	lnCLA	lnCTL	Geno	Lipid	Geno × lipid
% Composition							
16:0	15.45 ± 1.17	16.89 ± 0.54	17.28 ± 0.45	17.18 ± 0.44	NS	NS	NS
16:1n-7	0.88 ± 0.11	1.11 ± 0.07	0.32 ± 0.03	0.44 ± 0.08	<0.0001	<0.05	NS
17:0	0.34 ± 0.10	0.30 ± 0.03	0.43 ± 0.04	0.49 ± 0.06	<0.05	NS	NS
18:0	28.13 ± 0.91	26.74 ± 0.60	23.08 ± 0.50	24.49 ± 0.67	<0.0001	NS	NS
18:1n-9	2.72 ± 0.23[b]	3.36 ± 0.16[a]	2.80 ± 0.10[b]	2.59 ± 0.11[b]	0.0444	NS	<0.05
18:1n-7	1.26 ± 0.07	2.24 ± 0.23	1.39 ± 0.06	2.21 ± 0.07	NS	<0.0001	NS
18:2n-6	8.19 ± 0.36	6.97 ± 0.45	12.38 ± 0.17	10.57 ± 0.40	<0.0001	<0.01	NS
20:2n-6	0.30 ± 0.03	0.26 ± 0.03	0.37 ± 0.03	0.43 ± 0.03	<0.01	NS	NS
20:3n-6	1.66 ± 0.10[a]	1.11 ± 0.10[b]	0.52 ± 0.05[c]	0.54 ± 0.10[c]	<0.0001	<0.05	<0.01
20:4n-6	25.68 ± 1.44	27.45 ± 0.87	26.89 ± 0.32	27.46 ± 0.72	NS	NS	NS
22:0	0.26 ± 0.03	0.30 ± 0.06	0.28 ± 0.02	0.28 ± 0.05	NS	NS	NS
22:4n-6	0.45 ± 0.06	0.37 ± 0.06	0.31 ± 0.02	0.27 ± 0.03	<0.05	NS	NS
22:5n-3	1.32 ± 0.09	1.06 ± 0.08	0.95 ± 0.04	0.76 ± 0.03	<0.01	<0.01	NS
22:6n-3	8.06 ± 0.69	7.52 ± 0.44	7.58 ± 0.34	7.20 ± 0.54	NS	NS	NS
24:0	0.60 ± 0.04	0.62 ± 0.04	0.65 ± 0.04	0.64 ± 0.05	NS	NS	NS
24:1n-9	0.21 ± 0.04	0.34 ± 0.06	0.18 ± 0.01	0.21 ± 0.03	NS	NS	NS
Totals							
SFA	45.07 ± 0.41	45.18 ± 0.77	41.99 ± 0.22	43.47 ± 0.6	<0.01	NS	NS
MUFA	5.19 ± 0.40	7.17 ± 0.42	4.90 ± 1.18	5.68 ± 0.23	<0.05	<0.01	NS
PUFA	46.66 ± 1.93	45.09 ± 1.88	50.37 ± 0.22	47.68 ± 1.18	<0.05	NS	NS
SAT + MUFA + PUFA	96.92 ± 1.16	97.44 ± 1.20	97.25 ± 0.16	96.83 ± 0.79	NS	NS	NS
n-9	3.05 ± 0.28[b]	3.83 ± 0.16[a]	3.19 ± 0.10[b]	3.03 ± 0.10[b]	NS	NS	<0.05
n-6	36.26 ± 1.19	36.01 ± 1.39	40.39 ± 0.16	38.97 ± 0.67	<0.01	NS	NS
n-3	9.73 ± 0.69	8.82 ± 0.52	8.86 ± 0.37	8.28 ± 0.55	NS	NS	NS
Ratios							
MUFA/SFA	0.12 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	NS	0.0015	NS
PUFA/SFA	1.04 ± 0.05	1.00 ± 0.06	1.20 ± 0.01	1.10 ± 0.04	<0.01	NS	NS
n-9/n-6	0.09 ± 0.01	0.11 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	<0.05	NS	NS
n-6/n-3	3.78 ± 0.18	4.11 ± 0.13	4.60 ± 0.22	4.77 ± 0.25	0.0021	NS	NS
Δ^9 DS index[b]	0.08 ± 0.01[b]	0.10 ± 0.01[a]	0.07 ± 0.01[b]	0.07 ± 0.01[b]	<0.01	NS	<0.05

[a]Means ± SEM for $n = 5$ rats/group; main effects from ANOVA: Geno = genotype (*fa/fa* vs. lean rats), Lipid (0 vs. 1.5% CLA), and Geno × lipid = lipid × genotype interaction; means with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple range test; faCLA = *fa/fa* rats fed 1.5% CLA, faCTL = *fa/fa* rats fed 0% CLA, lnCLA = lean rats fed 1.5% CLA, lnCTL = lean rats fed 0% CLA; NS = not significantly different. Only FA present at >0.25% composition are reported.
[b]Δ^9 DS index = [(16:1n-7 + 18:1n-9)/(16:0 + 18:0)].

(isoform of Δ^9 desaturase). SREBP-1 and/or its target genes have been implicated in CLA-induced fatty liver in mice (7,8,33,35). This raises the question whether CLA amelioration of fatty liver in CLA-fed *fa/fa* rats involves inhibition of SREBP-1 pathway and/or its target genes. However, in the current study, CLA did not alter ACC, a SREBP target gene, in either genotype. It is possible that CLA could be affecting ACC phosphorylation and/or other enzymes of FA synthesis. For example, CLA supplementation decreased activity of other lipid synthesis enzymes including FA synthase *in vitro* (45) and hepatic microsomal phosphatidate phosphohydrolase in OLETF rats (10).

The current study shows that CLA decreases liver Δ^9 desaturase activity in both normal lean and obese insulin-resistant rats based on the FA composition of TAG and PL (Tables 4 and 5). SCD (+/+) mice have greater liver lipid content and circulating TAG, FFA, and cholesterol than SCD (–/–) mice (44). Furthermore, SCD1 deficiency prevents hepatic steatosis and hyperlidemia in PPARα-deficient mice. SCD1 plays a key role in hepatic TAG and VLDL synthesis, as 18:1 (product of SCD activity) is essential for TAG and cholesterol ester synthesis. Thus, in the present study, reduction of hepatic Δ^9 desaturase activity may be contributing in part to the mechanism for amelioration of hepatic steatosis and improvement in lipoprotein profile in CLA-fed *fa/fa* rats.

Other desaturase enzymes may also play an important role in mediating hepatic lipid levels. In the current study, *fa/fa* insulin-resistant rats had hepatic steatosis and lower percentages

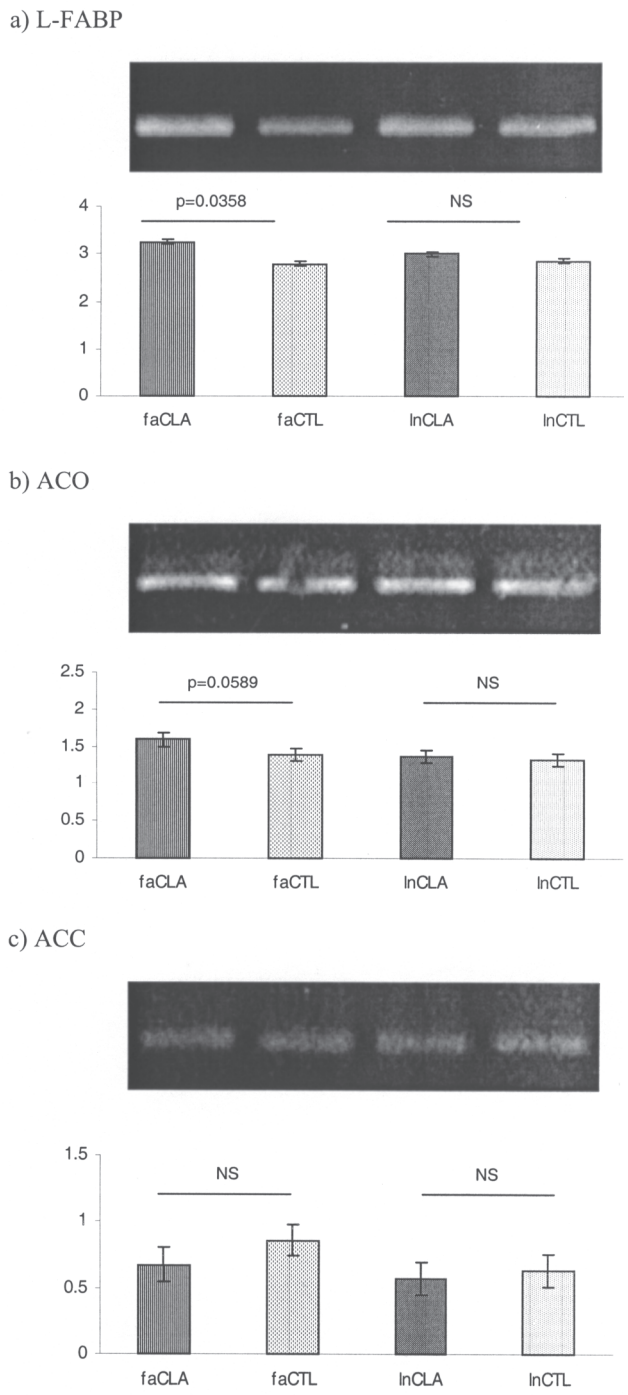

FIG. 5. Reverse transcription-polymerase chain reaction for mRNA levels of enzymes involved in (a) FA transport (L-FABP = liver FABP), (b) peroxisomal FA oxidation (ACO = acyl CoA oxidase), and (c) FA synthesis (ACC = acetyl CoA carboxylase). Results expressed as means for arbitrary units for $n = 10$ rats/group except $n = 9$ rats/group for L-FABP; *P*-values from preplanned comparisons using the housekeeping gene as a covariate; NS, not significantly different; for other abbreviations see Figure 1.

of longer-chain FA (18:3n-3, 20:4n-6, 20:5n-3, 22:5n-3, and 22:6n-3) in liver TAG, suggesting reduced activity of Δ^6-, Δ^5-desaturase, and/or enzymes of Sprecher's pathway compared with lean rats. Reduction of hepatic steatosis by CLA feeding in *fa/fa* rats was associated with a liver FA profile that resembled that of the lean rats. Investigations in mice support a role for impaired Δ^6 desaturation in the development of hepatic steatosis. Feeding 18:3n-6 (product of Δ^6-desaturation) plus CLA prevented the fatty liver normally induced by CLA in mice, possibly through altered eicosanoid metabolism (46). Thus, it is reasonable to speculate that amelioration of fatty liver by CLA in the *fa/fa* model was mediated by enhanced long-chain FA synthesis, with possible implications for eicosanoid production.

In summary, these results illustrate a strong relationship between the state of insulin resistance and liver lipid metabolism and suggest that CLA acts to favorably modify lipid metabolism in *fa/fa* Zucker rats, a model of insulin resistance and obesity. CLA reduced hepatic steatosis in the *fa/fa* Zucker rat, concomitant with reduced circulating ALT and alkaline phosphatase, improved lipoprotein profile, elevated markers of FA transport and oxidation, and altered liver FA profiles, thus implicating a role for altered liver desaturase activities.

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Selective Incorporation of Docosahexaenoic Acid into Lysobisphosphatidic Acid in Cultured THP-1 Macrophages

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ABSTRACT: Lysobisphosphatidic acid (LBPA) is highly accumulated in specific domains of the late endosome and is involved in the biogenesis and function of this organelle. Little is known about the biosynthesis and metabolism of this lipid. We examined its FA composition and the incorporation of exogenous FA into LBPA in the human monocytic leukemia cell line THP-1. The LBPA FA composition in THP-1 cells exhibits an elevated amount of oleic acid (18:1n-9) and enrichment of PUFA, especially DHA (22:6n-3). DHA supplemented to the medium was efficiently incorporated into LBPA. In contrast, arachidonic acid (20:4n-6) was hardly esterified to LBPA under the same experimental conditions. The turnover of DHA in LBPA was similar to that in other phospholipids. Specific incorporation of DHA into LBPA was also observed in baby hamster kidney fibroblasts, although LBPA in these cells contains very low endogenous levels of DHA in normal growth conditions. Our results, together with published observations, suggest that the specific incorporation of DHA into LBPA is a common phenomenon in mammalian cells. The physiological significance of DHA-enriched LBPA is discussed.

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During atherogenesis, peripheral blood monocytes traverse the arterial endothelium and differentiate into macrophages. Much of the vessel wall lesion of atherosclerosis is composed of macrophages that have become engorged with cholesterol (1,2). Cells acquire cholesterol by receptor-mediated endocytosis of LDL or oxidized LDL. After endocytosis, LDL and oxidized LDL are first transported to early endosomes and then late endosomes/lysosomes (3). The molecular mechanism of cholesterol transport from late endosomes/lysosomes is not well understood. The characteristic feature of the late endosome is the accumulation of internal membranes (4). Recently it was shown that these internal membranes are highly enriched with a unique phospholipid (PL), lysobisphosphatidic acid (LBPA), also called bis(monoacylglycerol) phosphate (5,6). Antibodies against LBPA, when internalized from the medium, alter both the organization of internal membranes and the membrane traffic from late endosomes (7,8). These results suggest that LBPA-rich internal membrane domains contribute to membrane sorting and/or trafficking from late endosomes. Accumulation of cholesterol in late endosomes in Niemann–Pick type C (NPC) cells and in drug-treated cells that mimic NPC impaired membrane traffic from late endosomes (9–11). Treatment of the cells with anti-LBPA antibody also resulted in a massive accumulation of cholesterol in late endosomes, suggesting that the characteristic network of LBPA-rich membranes contained within multivesicular late endosomes regulates cholesterol transport (9).

LBPA is a structural isomer of phosphatidylglycerol. Whereas phosphatidylglycerol binds two acyl groups to one glycerol moiety, LBPA binds one acyl group to each glycerol moiety. In addition, LBPA has an *sn*-1, *sn*-1′ glycerophosphate backbone, instead of *sn*-3 stereoconfiguration, which is a common structure of all other PL (12,13). The FA composition of LBPA varies among cell types. In baby hamster kidney (BHK) cells, more than 90% of FA in LBPA is oleic acid (OA; 18:1) (6,14) whereas pulmonary alveolar macrophages efficiently incorporate arachidonic acid (AA; 20:4) (15–17). DHA (22:6) is selectively incorporated into LBPA in rat uterine stromal cells (18). DHA attracts special attention since epidemiological, animal, and clinical studies all indicate that DHA has potent anti-inflammatory and antiatherogenic properties (19,20). It is reported that membranes enriched with DHA-containing PL exclude cholesterol (21–24). Phorbol ester-treated THP-1 monocytes are a widely used model in studying cholesterol homeostasis in human macrophages (25–27). In the present study, we characterized the content, distribution, and FA composition of LBPA in THP-1 cells. Our results indicate that LBPA selectively incorporates DHA into THP-1 cells. Our results also suggest that the preferential incorporation of DHA is a common phenomenon in LBPA.

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Abbreviations: AA, arachidonic acid; BHK, baby hamster kidney; EtnGpl, ethanolamineglycerophospholipid; INSA, Institut National des Sciences Appliquées; INSERM, Institut National de la Santé et de la Recherche Médicale; LBPA, lysobisphosphatidic acid; MUFA, monounsaturated FA; NPC, Niemann–Pick type C; OA, oleic acid; PL, phospholipids; PMA, phorbol myristate acetate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

MATERIAL AND METHODS

Materials. DHA and AA were from Sigma (Saint Quentin Fallavier, France). Tissue culture media were from Eurobio (Les Ulis, France). [9,10-^{3}H]OA (23 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol), [4,5-^{3}H]DHA (23 Ci/mmol), [1-^{14}C]DHA (54 mCi/mmol), and [1-^{14}C]EPA (20:5n-3) (54 mCi/mmol) were from PerkinElmer Life Science (Paris, France). All other lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Anti-Lamp-1 mouse monoclonal antibody was obtained from Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Anti-EEA1 mouse monoclonal antibody was from Transduction Laboratories (Lexington, KY). Anti-CD63 mouse monoclonal antibody was obtained from Cymbus Biotechnology (Southampton, United Kingdom). Alexa Fluor 488 conjugated anti-mouse IgG was from Molecular Probes (Eugene, OR). Anti-LBPA mouse monoclonal antibody was obtained as described (7). Alexa Fluor 546 conjugated anti-LBPA antibody was prepared according to the manufacturer's instruction (Molecular Probes).

Cell culture. Human monocytic leukemia cell line THP-1 was obtained from RIKEN Bioresource Center (Tsukuba, Japan). Cells were grown in RPMI 1640 medium containing 10% (v/v) FCS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in suspension in 75-cm^2 flasks at 37°C in an atmosphere of 5% CO_2 and subcloned periodically to maintain a cellular density around 2×10^5 cells/mL

Differentiation to a macrophage phenotype was induced by culturing 10–12 $\times$ 10^6 cells per 100-mm diameter dish in the presence of 100 nM phorbol myristate acetate (PMA) for 72 h. Where indicated, cells were differentiated in the presence of 5 μM AA or DHA. Medium was changed for the last 24 h. FA were added from ethanolic stock solutions, the final ethanol concentration being 0.025%. When radioactive FA were incorporated, cells were differentiated for 2 d in the presence of 100 nM PMA and then labeled with radioactive FA in complete medium for 24 h at 37°C in the presence of PMA. At the end of incubation, the medium was aspirated, cells were washed twice with PBS and scraped in PBS for lipid analysis. For immunofluorescence, cells were grown on glass-bottomed 35-mm dishes. Baby hamster kidney (BHK-21) cells were grown and maintained as described (28).

Lipid extraction and separation of lipids on TLC. Cells were collected by centrifugation and resuspended in methanol. Total lipids were extracted using the procedure of Folch *et al.* as described previously (18,29): 2 vol of chloroform were added to the methanolic cell suspension and after sonication and vortexing for 5 min, 0.9 vol of 0.88% KCl was added. Samples were vortexed and centrifuged for 10 min at 1500 $\times$ *g*. The chloroform layer was collected, and the aqueous phase was washed once with 2 vol of chloroform. The two chloroform layers were combined, dried under vacuum, and stored in chloroform at –20°C. Extracted lipids were spotted on a silica gel 60 TLC plate (20 $\times$ 20 cm, 0.25 mm; Merck AG, Darmstadt, Germany), which was developed in chloroform/methanol/40% methylamine (65:22:3, by vol). PL were revealed under UV light after spraying with 0.05% 2′,7′-dichlorofluorescein in methanol and were identified by comparison with authentic standards spotted on the same plate. For radioactive samples, the labeled compounds were detected and integrated using a Berthold LB 511 (Wildbad, Germany) TLC analyzer and identified by their R_f values as compared with those observed for standards. The silica gel containing lipids was scraped and lipids extracted using 2 $\times$ 2 mL chloroform/methanol/water (5:5:1, by vol). Water was added to the combined extracts to obtain 10:10:9 (by vol) chloroform/methanol/water mixture. The samples were vortexed and centrifuged, and the chloroform layer was collected. The solvent was then removed under nitrogen, and the dried lipids were resolubilized in chloroform.

HPLC. Purification of LBPA was carried out on a Nucleosil 5NH2, 250 $\times$ 4.6 mm column using a Hewlett-Packard 1100 HPLC system equipped with a quaternary pump and a diode array detector as previously described (18). The mobile phase was acetonitrile/methanol/water/50% methylphosphonic acid (730:250:15:0.3, by vol; pH adjusted to 6.2 with 28% NH_4OH) with a flow rate of 1.2 mL/min. The absorbance was monitored at 205 and 210 nm, and eluting lipids were identified by comparison with authentic standards. When radioactive lipids were analyzed, the detector was coupled with continuous-flow liquid scintillation counting using a Radiomatic Flow One β detector. The detector was operated with Ultima-Flow M (Packard) at a flow rate of 2.5 mL/min in a 400 μL cell. In some experiments, radioactive FAME were separated by reversed-phase HPLC using a 4.6 $\times$ 250 mm Ultrabase C18 column (SFCC-Shandon, Eragny, France) with 5 μm spherical particles as previously described (30,31). The mobile phase was acetonitrile/water (80:20 vol/vol), and the flow rate was 2.0 mL/min. The absorbance was monitored at 210 nm. Peaks were identified by comparing retention times with authentic standards.

GC. The FA composition of total lipids, PL classes, and purified LBPA was determined by GC after transmethylation using 5% H_2SO_4 in methanol (100°C, 90 min). FAME were analyzed with a Hewlett-Packard 5590 gas chromatograph. The capillary column was an SP2380 (0.32 mm, 30 m; Supelco, Bellefonte, PA). The temperature was programmed from 145 to 225°C at 1.2°C/min. Helium was used as a carrier gas. FAME were identified by comparison with the relative retention times of known standards. The percentage and mass of each FA were calculated using an internal standard (pentadecanoic acid methyl ester) as described previously (18,30).

Immunofluorescence. All manipulations were performed at room temperature. Cells were first washed with PBS and then were fixed with 3% paraformaldehyde in PBS for 20 min, quenched with 0.1 M NH_4Cl and then blocked with 0.2% gelatin in PBS. Cells were then permeabilized by treatment with 50 μg/mL digitonin for 5 min followed by incubation with the first monoclonal antibodies. After 30 min of incubation with Alexa Fluor 488 conjugated anti-mouse IgG, cells were washed with PBS and further incubated with Alexa Fluor 546 conjugated anti-LBPA monoclonal antibody. The specimens were mounted with Mowiol and examined under a Zeiss LSM

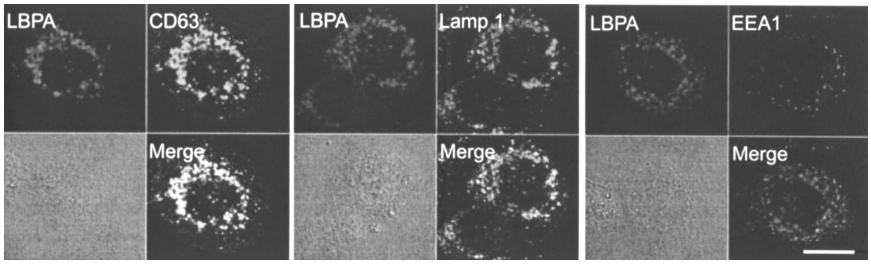

FIG. 1. Intracellular distribution of lysobisphosphatidic acid (LBPA) in THP-1 macrophages examined by immunofluorescence. THP-1 cells were grown for 3 d in the presence of 100 nM phorbol myristate acetate (PMA). Cells were then fixed and permeabilized as described in the Materials and Methods section. Cells were then doubly labeled with anti-LBPA antibody and various endosome markers as indicated. Bar, 10 μm.

510 confocal microscope equipped with C-Apochromat 63XW Korr (1.2 n.a.) objective.

Data analysis. Data are expressed as mean ± SD. Statistically significant difference was tested using Student's *t*-test for paired samples.

RESULTS

LBPA in THP-1 cells. In the present study, we characterized LBPA in the cultured macrophage cell line THP-1. LBPA was purified from THP-1 total lipid extracts, using a first separation from total lipids by TLC, and a further separation by HPLC as described previously (18). The proportion of LBPA compared with other PL was determined on the basis of FA content in the different PL classes. Expressing the results as a percentage of total PL, based on the FA amount in each PL class, LBPA represents 1.4 ± 0.7% (mean ± SD) of the total PL, phosphatidylcholine (PtdCho) 51.7 ± 3.5%, phosphatidylethanolamine (PtdEtn) 23.8 ± 7.0%, sphingomyelin 6.0 ± 1.3%, and phosphatidylinositol/phosphatidylserine 16.3 ± 2.3%. The low proportion of LBPA was also found in THP-1 cells before differentiation into macrophages (data not shown).

The intracellular distribution of LBPA was then measured using a specific monoclonal antibody (7). In Figure 1, THP-1 macrophages were doubly labeled with anti-LBPA and anti-CD63 monoclonal antibodies, anti-LBPA and anti-Lamp 1 monoclonal antibodies, or anti-LBPA and anti-EEA1 monoclonal antibodies, as described in the Materials and Methods section. CD63 is a member of the four-time membrane-spanning proteins (tetraspanins) and is enriched in the internal membranes of multivesicular late endosomes (32,33). Lamp-1 is localized in the limiting membranes of late endosomes (7,34). LBPA was not co-localized with the early endosome marker, EEA1 (35). Co-localization of LBPA with late endosome markers indicates that, similar to other cell types (7,36), LBPA is enriched in late endosomes in THP-1 macrophages.

TABLE 1
FA Composition (%mol) of THP-1 Macrophages Unsupplemented and Supplemented with 5 μM Arachidonic Acid (AA) or DHA[a]

	PtdCho			PtdEtn			LBPA		
		Supplemented			Supplemented			Supplemented	
FA	Control	5 μM DHA	5 μM AA	Control	5 μM DHA	5 μM AA	Control	5 μM DHA	5 μM AA
16:0	35.6 ± 0.3	40.3 ± 3.6	43.2 ± 1.9*	14.6 ± 1.1	17.9 ± 1.5	25.7 ± 0.4**	23.1 ± 2.4	22.8 ± 2.15	28.6 ± 3.9
16:1	12.7 ± 3.3	10.4 ± 1.9	6.3 ± 0.6*	9.6 ± 1.4	7.5 ± 1.2	2.5 ± 0.3*	6.4 ± 1.2	8.7 ± 0.7	4.4 ± 1.3
18:0	4.5 ± 1.7	4.4 ± 0.8	5.0 ± 0.4	11.4 ± 4.6	13.2 ± 2.2	17.1 ± 2.3	24.1 ± 12.1	9.6 ± 2.1	16.4 ± 4.3
18:1	37.6 ± 2.5	32.8 ± 5.7	28.2 ± 0.9*	27.5 ± 0.7	25.2 ± 1.4	18.1 ± 0.4**	26.5 ± 6.8	17.5 ± 1.8	15.6 ± 6.0
18:2n-6	1.6 ± 0.2	1.6 ± 1.4	3.7 ± 3.1**	3.0 ± 0.9	3.6 ± 1.8	4.8 ± 2.1	4.7 ± 2.7	3.2 ± 1.1	4.1 ± 2.3
20:4n-6	1.5 ± 0.3	1.5 ± 0.4	5.1 ± 0.1**	9.7 ± 2.7	8.7 ± 1.0	18.7 ± 0.3*	7.2 ± 0.5	1.0 ± 1.3**	4.3 ± 3.1
20:5n-3	Trace	Trace	1.1 ± 0.2	2.1 ± 0.9	4.4 ± 1.5	4.0 ± 0.3	Trace	Trace	Trace
22:5n-3	Trace	Trace	Trace	1.2 ± 1.7	1.2 ± 0.2	1.6 ± 0.2	Trace	Trace	Trace
22:6n-3	0.8 ± 0.0	2.4 ± 1.4*	1.1 ± 0.2	5.2 ± 0.6	14.8 ± 1.7**	3.6 ± 0.1	6.6 ± 1.6	29.3 ± 2.1**	9.5 ± 2.3
Saturated	44.4 ± 1.4	49.4 ± 3.0	52.3 ± 2.8*	30.3 ± 3.6	32.4 ± 2.0	43.2 ± 3.3	47.2 ± 9.7	39.6 ± 5.4	54.9 ± 5.3
MUFA	50.8 ± 15	43.5 ± 7.1	34.7 ± 1.4	42.6 ± 5.2	33.1 ± 1.6	20.7 ± 0.1*	32.8 ± 5.6	24.8 ± 3.3	21.5 ± 5.8
PUFA (n-6)	3.6 ± 0.5	3.5 ± 2.1	10.7 ± 3.5*	16.4 ± 3.8	14.0 ± 2.1	26.9 ± 3.2*	13.4 ± 2.5	4.2 ± 2.2**	10.4 ± 2.1
PUFA (n-3)	1.1 ± 0.4	3.7 ± 2.0	2.5 ± 0.3**	9.8 ± 0.8	20.5 ± 2.5*	9.3 ± 0.0	6.6 ± 1.6	31.4 ± 2.6**	12.1 ± 3.6

[a]Total lipids were obtained from THP-1 macrophages [3 d of differentiation with 100 mM of phorbol myristate acetate (PMA)] without (control cells) or with 5 μM of FA. Phospholipids (PL) were separated by TLC and analyzed as described in the Materials and Methods section. The FA composition is expressed as mol% of total FA, and represents means ± SD from three separate experiments. PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; LBPA, lysobisphosphatidic acid; MUFA, monounsaturated FA. Trace means <1% of total FA. Significant differences with respect to the values of unsupplemented cells were determined using Student's *t*-test and are indicated with asterisks. *$P < 0.05$; **$P < 0.01$.

TABLE 2
Percent Distribution[a] of Radioactivity in Lipids from THP-1 Macrophages Labeled for 24 h with [^{3}H]AA, [^{3}H]OA, [^{14}C]DHA, or[^{14}C]EPA

	Percentage of total radioactivity in PL			
Radioactive FA	ChoGpl	EtnGpl	PtdIns/PtdSer/Cer*P*Cho	LBPA
DHA	19.5 ± 4.2	66.3 ± 4.1	6.5 ± 3.0	6.4 ± 1.1
AA	19.0 ± 2.7	69.4 ± 7.5	13.6 ± 0.8	0.8 ± 0.3
EPA	16.1 ± 2.7	72.7 ± 8.5	9.7 ± 2.2	1.6 ± 0.4
OA	63.8 ± 0.1	21.8 ± 0.1	4.2 ± 0.3	7.5 ± 0.1
DHA/AA	1.0	1.0	0.5	8.0

[a]Values refer to averages ± SD of three different experiments. Total lipids were obtained from THP-1 cells differentiated 2 d with 100 nM of PMA and the last 24 h, with 100 nM PMA plus 0.5 μCi/mL of radioactive FA. PL classes were separated by TLC and analyzed as described in the Materials and Methods section. OA, oleic acid; ChoGpl, cholineglycerophospholipids; EtnGpl, ethanolamineglycerophospholipids; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; Cer*P*Cho, sphingomyelin; for other abbreviations see Table 1.

LBPA is enriched with DHA in THP-1 cells. Table 1 shows the FA composition of LBPA and two major PL classes, PtdCho and PtdEtn, in THP-1 macrophages. The FA composition of PtdCho and PtdEtn obtained in this experiment is close to that reported by others (37). PtdEtn contains a high level of monounsaturated FA (MUFA) and is enriched with PUFA compared with PtdCho, which is mainly constituted of saturated FA and MUFA. The major FA in LBPA were saturated FA and MUFA, especially 18:1, which represents 26% of the total FA. However, the PUFA content in LBPA was relatively high. n-3 and n-6 PUFA together account for 20% of the total FA in LBPA, whereas this proportion was 26% in PtdEtn and less than 5% in PtdCho. The main PUFA of LBPA were AA (20:4n-6) and DHA (22:6n-3), which were found in similar proportions. It is noteworthy that DHA accounts for 33% of the total PUFA in LBPA. This percentage is higher than those in PtdEtn and PtdCho (20 and 17%, respectively) whereas AA represents around 35% of the total PUFA regardless of the PL class. Moreover, DHA was the only n-3 PUFA representative in LBPA. In contrast, PtdEtn contained both EPA (20:5n-3) and docosapentaenoic acid (22:5n-3) in addition to DHA. We have also analyzed PL from undifferentiated THP-1 cells (data not shown) and found similar FA compositions, indicating that PMA treatment and the differentiation of monocytes into macrophages had no significant effect on cellular FA composition.

DHA is selectively incorporated into LBPA in THP-1 cells. We then asked whether exogenously added FA alter FA composition of LBPA, PtdCho, and PtdEtn. As shown in Table 1, after DHA supplementation, the proportion of this FA was significantly increased in the three PL compared with their respective controls. Interestingly, the highest increase of DHA was found in LBPA with a 4.5-fold increase vs. a 3- and 2.8-fold increase, respectively, in PtdCho and PtdEtn. This increase of DHA in LBPA was compensated for by a significant decrease in the proportion of AA and a less marked decrease of stearic acid (18:0) and OA. In contrast, incorporation of DHA did not significantly decrease the percentage of AA in PtdCho and PtdEtn. In supplemented cells, DHA accounted for more than 90% of the total n-3 PUFA in LBPA as compared with 70% in PtdEtn. Determination of the FA amounts revealed that the cellular LBPA content was not affected by incubation with DHA (0.26 ± 0.12 vs. 0.27 ± 0.12 nmol/10^6 cells, in control and supplemented cells, respectively). After AA supplementation, the proportion of AA was significantly increased in PtdCho and PtdEtn, and levels of linoleic acid were also increased, with a compensatory decrease in MUFA. In contrast, no increase of AA and its retroconversion products was observed in LBPA.

TABLE 3
Percent Distribution[a] of [^{14}C]DHA, or[^{3}H]DHA in PL from THP-1 Macrophages Coincubated with 5 μM AA

	Percentage of total radioactivity in PL			
FA	ChoGpl	EtnGpl	PtdIns/PtdSer/Cer*P*Cho	LBPA
[^{14}C]DHA 5 μM	23.6 ± 4.2	64.5 ± 4.7	4.7 ± 0.7	7.2 ± 0.6
+ AA 5 μM	24.0 ± 2.7	63.7 ± 3.1	4.2 ± 0.2	8.0 ± 0.1
[^{3}H]DHA 10 nM	22.3 ± 0.7	61.9 ± 0.8	7.0 ± 0.3	8.8 ± 0.8
+ AA 5 μM	25.0 ± 1.6	58.8 ± 1.5*	6.9 ± 0.3	9.3 ± 0.7

[a]Values refer to averages ± SD of triplicates. Total lipids were obtained from THP-1 cells differentiated 2 d with 100 nM of PMA and the last 24 h, with 100 nM PMA plus 5 μM [^{14}C]DHA or 10 nM [^{3}H]DHA in the absence or presence of 5 μM AA. PL were separated by TLC and analyzed as described in the Materials and Methods section. For abbreviations see Tables 1 and 2.

TABLE 4
Percent Distribution[a] of Radioactivity in PL from BHK Cells Labeled for 24 h with [^{3}H]AA, [^{3}H]OA, or [^{3}H]DHA

	Percentage of total radioactivity in PL			
Radioactive FA	ChoGpl	EtnGpl	PtdIns/PtdSer/Cer*P*Cho	LBPA
DHA	5.8 ± 0.8	76.1 ± 4.2	13.9 ± 3.6	3.0 ± 0.6
AA	7.0 ± 0.6	70.3 ± 0.9	21.3 ± 0.1	0.4 ± 0.1
OA	38.7 ± 2.6	45.8 ± 3.7	10.7 ± 2.1	4.5 ± 0.5
DHA/AA	0.8	1.1	0.7	7.5

[a]Values refer to averages ± SD of three different experiments. Total lipids were obtained from baby hamster kidney (BHK) cells labeled for 24 h with 0.5 μCi/mL of radioactive FA. PL were separated by TLC and analyzed as described in the Materials and Methods section. For other abbreviations see Tables 1 and 2.

Altogether, these results show that DHA is preferentially incorporated into LBPA.

The apparent selectivity of LBPA for DHA was further examined by comparing the incorporation of several radioactive FA into PL classes. Radiolabeled OA, AA, EPA, and DHA were incubated with THP-1 macrophages for 24 h, and the distribution of cellular radioactivity was determined after separation of the lipid and PL classes by TLC (Table 2). For OA and DHA, nearly 5% of the incorporated radioactivity was found associated with LBPA, which represents less than 1% of the total PL in terms of mass. This is indicative of a high rate of incorporation of these FA in LBPA, when compared with AA. Indeed, DHA was eightfold more efficiently incorporated than AA into LBPA (Table 2). After incubation with EPA, a precursor of DHA, LBPA was only slightly labeled. EPA was metabolized to DHA, with 2% of radioactive EPA being converted to DHA as shown in total cellular lipids. This proportion increased up to 40% in LBPA (data not shown), indicating that DHA, which was derived from EPA, was preferentially esterified into LBPA. These results, together with those presented in Table 1, show that regardless of the source of DHA (i.e., formed either by elongation/desaturation or by direct addition), DHA was incorporated selectively into LBPA in THP-1 macrophages.

We next examined whether the selectivity for DHA still would be observed if DHA was coincubated with AA. This condition mimics the *in vivo* situation where both FA are present at comparable levels and could therefore compete for uptake and incorporation mechanisms. THP-1 macrophages were incubated with 5 μM [^{14}C]DHA (0.3 μCi/mL) in the absence or presence of 5 μM AA. Total incorporation of radioactive DHA was not affected by the presence of exogenous AA (92.8 ± 0.06% of initially added radioactivity vs. 92.9 ± 0.21% in the control experiment). In addition, the distribution of incorporated radioactivity to total PL was not significantly altered (85.3 ± 5.1% of cellular radioactivity vs. 87.6 ± 4.4% in the control). As shown in Table 3, the percentage of DHA incorporated into LBPA was not modified in the presence of 5 μM AA. We have also incubated macrophages with 10 nM [^{3}H]DHA (0.3 μCi/mL) together with a 500 times higher concentration of AA (5 μM). In this condition, coincubation with AA reduced the cellular uptake of DHA (83.6 ± 2.5% of initially added radioactivity vs. 95.2 ± 5.2% in the control, $P \leq 0.05$) but not its distribution in total PL (82.2 ± 1.8% of cellular radioactivity vs. 85.3 ± 3.7% in controls). Of interest, the percentage of DHA incorporated into ethanolamineglycerophospholipids (EtnGpl) was slightly decreased whereas it was not changed into LBPA (Table 3). These results indicate the LBPA selectivity for DHA even when a high level of AA is available for incorporation.

DHA is selectively incorporated into LBPA in BHK cells. Selective incorporation of DHA into LBPA has been reported in several cell types (18,38). The results in pulmonary macrophages are controversial. Whereas Huterer and Wherrett (38) showed the efficient incorporation of DHA, Waite and colleagues (17) reported that LBPA is a reservoir of AA. The FA composition of LBPA varies among cell types. In BHK cells, more than 90% of FA in LBPA is OA (18:1) (6). To determine whether selective incorporation of DHA into LBPA is a common phenomenon, we measured the incorporation of radioactive FA in BHK cells. Radioactive DHA and OA were efficiently incorporated into LBPA whereas, as observed in THP-1 cells, AA was not significantly incorporated with a DHA/AA ratio of around 8, as shown in Table 2 for THP-1 cells (Table 4).

DHA turns over similarly in PtdCho, PtdEtn, and LBPA in THP-1 cells. The present results, together with previous reports, confirm the selective ability of LBPA to incorporate DHA and suggest that this selectivity is extended to most cell types, regardless of their DHA content in LBPA. The reason for the preferential incorporation of DHA into LBPA is not understood. It has been suggested that the turnover of certain FA, such as AA and OA, is high in LBPA of pulmonary alveolar macrophages (15). Conversely, one might suggest that the turnover of DHA in LBPA is particularly slow compared with that in other PL, allowing its accumulation in that pool. This possibility was examined in THP-1 cells by following the removal of DHA from PL after the cells had been supplemented with DHA. As shown in Figure 2, after 3 d of supplementation, the DHA content was increased by 400% in LBPA, 280% in PtdEtn, and 220% in PtdCho compared with nonsupplemented control cells. After DHA removal, the DHA content decreased progressively back to the initial value in all PL. The data show no significant difference of DHA turnover in LBPA compared with PtdCho and PtdEtn.

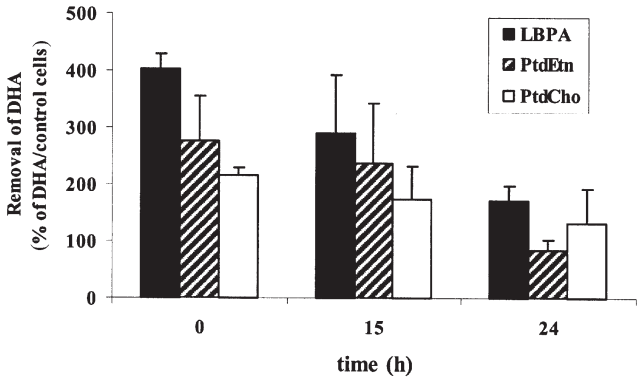

FIG. 2. Turnover of DHA in various phospholipids in THP-1 macrophages. THP-1 cells were grown for 3 d in the presence of 100 nM PMA and 5 μM DHA (time 0). DHA was then removed and cells were further incubated for various intervals in normal medium. Lipids were then extracted, and DHA composition was measured by GC. DHA content without supplementation represents 100%. PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; for other abbreviations see Figure 1.

DISCUSSION

The present study was undertaken to characterize LBPA in THP-1 macrophages in the context of related investigations on the putative role of LBPA in cholesterol homeostasis. We first showed that LBPA is almost exclusively localized in late endosomes in THP-1 macrophages, which is important regarding its involvement in LDL-derived cholesterol transport. Lipid composition of monocytic THP-1 cells already has been reported, but no data were available for differentiated macrophages, in particular for LBPA content and FA composition. Our data indicate that LBPA represents 1–2% of total PL in THP-1 macrophages and accumulates in late endosomes. This proportion is not different from peritoneal macrophages in which LBPA was also reported to be a minor PL (15). In contrast, in pulmonary alveolar macrophages, LBPA constitutes roughly 15–18% of the total cellular PL (15,16). It has been postulated that the presence of a large quantity of the precursor phosphatidylglycerol in lung surfactant could account for such a high level of LBPA in alveolar macrophages (39). Most other mammalian cell types, including human fibroblasts (39), polymorphonuclear leukocytes (16), BHK cells (6,14), PC12 cells (40), and rat uterine stromal cells (18) contain low amounts of LBPA. A striking accumulation of LBPA, however, is found in tissues from patients suffering from genetic lipidosis such as Niemann–Pick disease (41) as well as rat organs treated with drugs that induced lysosomal lipid storage (14,42–44).

The FA composition of LBPA in THP-1 macrophages resembles that of other cell types in terms of the elevated amount of OA (18:1n-9) and enrichment in PUFA. The proportion of OA was found to constitute 30–50% of total FA of LBPA in uterine stromal cells (18) and pulmonary alveolar macrophages (15–17), reaching more than 80% in BHK cells (6,14). With respect to PUFA, our data suggest an enrichment in DHA, as reported for PC12 cells (40) and uterine stromal cells (18). This is strikingly different from the situation observed in pulmonary alveolar macrophages. The latter cells contain mostly n-6 FA, mainly linoleic acid (18:2n-6) and AA, but have only a trace amount of DHA in their LBPA (15–17). In rat testis, 22:5n-6 accounts for more than 70% of the total FA of LBPA (18). It thus appears that while PUFA are consistently reported as being substantial constituents of LBPA, the specific composition of the PUFA varies depending on the cell type. This supports the idea that FA composition of LBPA could depend on the cellular function of this PL, as described for AA-containing LBPA in rabbit pulmonary macrophages (17).

Our results show that both THP-1 and BHK cells efficiently incorporated DHA into LBPA by comparison with other FA such as OA and AA. It should be noticed that although the percentage of DHA incorporation was not significantly different from that of OA in BHK cells, OA represents more than 80% of the endogenous FA in LBPA of those cells. In contrast to DHA, incorporation of AA into LBPA was very inefficient in both THP-1 and BHK cells. Preferential incorporation of DHA into LBPA has been reported in other cell types. In pulmonary alveolar macrophages, DHA was more efficiently incorporated than AA into LBPA despite a much lower endogenous content of DHA (0.5 vs. 7%) (38). In uterine stromal cells, where AA makes up about 6% of the total FA in LBPA (18), no labeling was detected after incubation with radioactive AA, while DHA was readily incorporated. Our present data further show that the high incorporation of DHA into LBPA was not affected by the presence of a high level of AA, which brings out further evidence for selectivity of LBPA toward DHA. One interesting point is that, whereas AA and DHA were found in similar amounts in LBPA in THP-1 macrophages, only exogenous DHA, but not AA, was incorporated in this PL. This suggests that DHA turnover in LBPA is much more rapid than that of AA. The specific localization of LBPA in late endosomes may also be important in this respect. It is possible that in these acidic compartments, FA metabolism differs from what is typically described for cellular membrane PL. For example, it is not known whether all FA can equally reach these compartments or whether classical acyltransferases are efficient. This is an important difference with EtnGpl that are enriched in both n-3 and n-6 PUFA and can also incorporate AA at a high rate. It should be noted that both EtnGpl and LBPA show high affinity for DHA, which raises the question of a potential relationship between these PL. Preliminary data using plasmalogen EtnGpl-deficient human skin fibroblasts showed that the DHA content in LBPA was not changed in these cells compared with controls, arguing against a role of plasmalogens as a reservoir of DHA during LBPA remodeling. From our present data, we can exclude a difference in turnover of DHA in LBPA compared with other PL to explain DHA enrichment and high incorporation. It may be alternatively proposed that the selective incorporation of DHA into LBPA reflects a DHA-specific transacylase activity (13,45) or is related to the unique cellular localization of LBPA. Further experiments will be necessary to elucidate the molecular mechanisms of the preferential incorporation of DHA into LBPA.

The physiological relevance of DHA enrichment of LBPA must also be addressed. LBPA is almost exclusively localized in membranes of late endosomes that are essential compartments of the endocytic pathway and participate in LDL-derived cholesterol distribution. The content of DHA in biological membranes has been shown to influence membrane properties such as fluidity, fusion, and raft formation (24). Importantly, it has been reported that membranes enriched with DHA-containing PL exclude cholesterol (21–23). These properties may be advantageous for the removal of cholesterol from late endosomes. DHA has interesting medical implications since its dietary presence has been positively linked to the prevention of numerous human afflictions including cancer and heart disease (46–49). However, little is known about the molecular mechanisms of action of DHA. Future study of DHA in LBPA may shed a revealing light on the biological role of this unique FA.

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METHOD

Characterization of Hair Lipid Images by Argon Sputter Etching–Scanning Electron Microscopy

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ABSTRACT: Hair lipid images, as visualized by argon sputter etching–scanning electron microscopy (ASE–SEM), reveal convex structures with a stitch pattern (SP) at the cell membrane complex (CMC) in the transverse hair plane. Based on interindividual variation, different features of the convex SP were classified into Types 0 to 4 with the corresponding scores 0 to 4. Observations using hair fibers collected from 27 Japanese females revealed significant positive correlations between the scores and the levels of exogenous lipids, which suggests that exogenous lipids internalized at the CMC predominantly constitute the convex SP. Intraindividual variation with different levels of exogenous lipids among hair fibers derived from individual females may be relevant to the uneven physicochemical properties of hair fibers on the scalp. Observations of 380 hair fibers collected from Japanese (Mongoloid), German and American (Caucasoid) females aged 3 to 77 yr demonstrated similar age-related changes in the lipid images, which represent an increase and then a decrease in levels of exogenous lipids with increasing age. This suggests that age-related changes in exogenous lipids are attributable to alterations in sebum excreted during aging and that this elicits age-related changes in physical parameters, which affect human hair texture.

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Human hair fibers are composed mainly of proteins (>90% dry hair weight) and lipids (1–9%). Each hair fiber consists of a central medulla surrounded by more tightly packed, spindle-shaped cortical cells, with 5 to 10 layers of flat, overlapping scale-like cuticle cells and with the cell membrane complex (CMC) located in the intercellular spaces between cuticle and cortical cells (1). Structures such as the cuticle, the cortex, the medulla (absent in some fibers), and the CMC are common among hair fibers of different races (2–5), although their diameters, shapes, and colors are different (1,6). Squalene (SQ), wax esters (WEs), triglycerides (TGs), free fatty acids (FFAs), cholesterol (CH), ceramides (CERs), and cholesterol sulfate (CS) comprise the free lipids, whereas 18-methyl eicosanoic acid is a bound lipid within hair fibers (7–14). Since hair lipids exist at the CMC and at the cuticle surface, they seem to play roles in the physicochemical characteristics of hair fibers, including the chemical diffusion barrier, the water-holding property, and the cell cohesion (14–17). The composition of hair lipids is changed by effects of daily weathering such as shampooing, conditioning, exposure to sunlight, and brushing (18), and by chemical oxidation such as bleaching (Masukawa, Y., Tsujimura, H., Tanamachi, H., Narita, H., and Imokawa, G., unpublished data). However, the reports of age-related changes in hair lipids are few (16,19,20).

We established a novel method for visualizing hair lipids at the CMC using argon sputter etching–scanning electron microscopy (ASE–SEM) (21). This method provides characteristic images consisting of a circular pattern (CP) and/or a stitch pattern (SP) at the cortex in a transversely polished hair plane, without complicated procedures such as are needed for transmission electron microscopy (2–5). The CP and the SP are formed at melanin granules and the CMC, respectively, as convex structures. The convex formation of the SP is ascribed to CMC lipids, which may ooze out from the inside to the surface of the hair plane as a result of the heat generated during the ASE and which may be chemically changed into immobilized structures as a part of the CMC. This effect allows us to characterize the distribution of hair lipids at the CMC using the novel ASE–SEM methodology (21). However, it remains to be clarified which lipids among free hair lipids [such as SQ, WEs, TGs, FFAs, CH, CERs and CS (7–13)] are the predominant contributors to the convex SP in ASE–SEM images of hair fibers.

With this new methodology (21), the variation of the convex SP in ASE–SEM images was examined in this study by using hair fibers from 27 Japanese females, and the ASE–SEM images were then classified and scored based on the frequency and strength of the convex SP. These scores were compared with the levels of hair lipids. Further, age-related changes in the convex SP in ASE–SEM images were evaluated using 380 hair fibers from Japanese (Mongoloid), German and American (Caucasoid) females (3–77 yr old). We now report that higher levels of exogenous lipids within hair fibers predominantly induce greater convex SP and that the resulting hair lipid profiles change with aging in all races tested.

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Abbreviations: ASE, argon sputter etching; BC, β-cholestanol; CER/MS, ceramide/*N*-myristoyl sphingosine; CER/PDS, ceramide/*N*-palmitoyl-DL-dihydrosphingosine; CERs, ceramides; CH, cholesterol; CMC, cell membrane complex; CP, circular pattern; CS, cholesterol sulfate; FFAs, free fatty acids; SEM, scanning electron microscopy; SIM, selected ion monitoring; SP, stitch pattern; SQ, squalene; TGs, triglycerides; WEs, wax esters.

MATERIALS AND METHODS

Chemicals. Epon 812 resin, 25% glutaraldehyde, and osmium tetroxide were purchased from TAAB (Reading, United Kingdom). As authentic lipids, SQ, palmityl palmitate, tripalmitin, palmitic acid, CH, β-cholestanol (BC), and CS were purchased from Sigma (St. Louis, MO). Ceraminde/*N*-palmitoyl-DL-dihydrosphingosine (CER/PDS) and ceramide/*N*-myristoyl sphingosine (CER/MS) were from Funakoshi (Tokyo, Japan) and from Avanti Polar Lipids (Alabaster, AL), respectively. Hexane, chloroform, methanol, and pyridine of infinity pure grade were from Wako Pure Chemicals Industries (Tokyo, Japan). Methanol of HPLC grade was from Kanto Reagents (Tokyo, Japan) and was used to prepare the mobile phase solution for LC connected to MS analysis. *N*-Trimethylsilylimidazole was from GL Science (Tokyo, Japan) and was used to derivatize hair lipids. Ultra-pure water prepared using a Milli-Q purification system (Millipore, Bedford, MA) was used for all analytical procedures. All other chemicals were of the highest analytical grade commercially available.

Materials. Scalp hair fibers from the proximal root end were kindly provided by 27 Japanese females (designated as No. 1 to No. 27) who had not treated their hair with chemical reactions such as permanent waving, bleaching, or coloring for at least 6 mon. In a second experiment, a total of 380 hair fibers, each of which was cut just at the proximal root end for each individual, were collected from 380 females [153 Japanese (Mongoloid) ranging from 3 to 68 yr old, 169 Germans (Caucasoid) from 18 to 77 yr old, and 58 Americans (Caucasoid) from 10 to 65 yr old]. None of the 380 females had treated their hair with chemical reactions such as permanent waving, bleaching, or coloring for at least 2 mon before collection.

For observations using ASE–SEM, hair fibers were washed with *n*-hexane for 5 min to remove the surface lipids and then were air-dried. In contrast, in preparation for analyzing hair lipid contents, hair fibers (*ca*. 20 mg) were washed successively with plain shampoo for 1 min and with tap water for 10 min. This procedure was repeated twice. The fibers were then dried at room temperature for more than 48 h, and, after washing with *n*-hexane for 5 min, their weight was measured accurately. The washing with *n*-hexane for 5 min was determined based on our previous experiment on the effects of *n*-hexane incubation time of hair fibers on extracted lipid contents (20), which suggested that the lipids present on the inside of the hair are removed under *n*-hexane incubation for more than 5 min.

ASE–SEM observations. In all hair fibers, the region 1-cm distant from the proximal root end was observed by ASE–SEM according to previously reported procedures (21). Thus, each hair fiber was embedded in Epon 812 resin, and each resin-embedded hair block was transversely polished with a diamond knife (MT6740; DiATOME Ltd., Biel, Switzerland) and was exposed to ASE (Hitachi Ion Sputter E-1030, Hitachinaka, Japan). Each specimen was then etched under conditions of a pressure of 6 to 7 Pa, a current of 6 mA, a voltage of 0.3 kV, a working distance of 30 mm, and a time of 360 s. After coating with Pt-Pd using the same instrument (Hitachi Ion Sputter E-1030), the polished hair planes were observed using a Hitachi S-4300 field emission scanning electron microscope with an accelerating voltage of 5 kV.

Analysis of hair lipids. Hair lipids were analyzed as previously reported (18). All analyses were performed in duplicate, and their average values are expressed as mg/g hair. Small pieces of hair fibers were cut with scissors and were immersed successively in chloroform/methanol (2:1, 1:1, 1:2, vol/vol) and chloroform/methanol/water (18:9:1, by vol) for 24 h. Each filtrate of solvent mixtures was dried under a nitrogen stream, and the residue was then dissolved in chloroform/methanol. Although there are several molecular species of CERs in human hair (12), CER/PDS was determined as a representative target because it is one of the predominant molecules in the hair fibers.

Levels of SQ, WEs, TGs, and FFAs were determined by TLC coupled to FID, using an Iatroscan MK-5 analyzer (Iatron Laboratories, Tokyo, Japan), a silica gel-coated Chromarod SIII (Iatron Laboratories), and a microdispenser (Drummond Scientific Co., Broomall, PA,). The level of CS was determined by LC–MS using an Agilent 1100 Series LC/MSD SL system equipped with ChemStation software (Agilent Technologies, Palo Alto, CA) and an L-Column ODS 2.1 mm i.d. × 150 mm (Chemical Evaluation and Research Institute, Tokyo, Japan). The mass spectrometer, using electrospray ionization, was operated in selected ion monitoring (SIM) mode with $m/z = 465$ for CS. Levels of CH and CER/PDS followed by trimethylsilylation were determined by GC–MS using an Agilent 6890 Series GC coupled to an Agilent 5973 mass selective detector, equipped with ChemStation software (Agilent Technologies) and an Ultra Alloy #1 metal capillary column of 30 m × 0.25 mm i.d. and a film thickness 0.15 μm. The internal standards BC and CER/MS were added to the lipid samples containing CH and CER/PDS, and they were then trimethylsilylated. The mass spectrometer was operated in electron impact mode, and the ions $m/z = 443$, 445, 313, and 342 in SIM were detected for trimethylsilylated-CH, trimethylsilylated-BC, trimethylsilylated-CER/PDS, and trimethylsilylated-CER/MS, respectively.

Statistical analyses. Correlation coefficients between the scores of the ASE–SEM images and each level for hair lipids were determined using Excel Tahenryo-Kaiseki version 3.0 software (Esumi, Tokyo, Japan).

RESULTS

ASE–SEM images of hair fibers from Japanese females. Representative ASE–SEM images of a transversely polished hair plane are shown in Figure 1. In SEM images without ASE, the hair plane can be visualized only as an even surface; when ASE treatment is performed on the hair plane prior to SEM observation, a characteristic image, consisting of the convex CP that reflects melanin granules and the convex SP that reflects lipid portions of the CMC at the cortex, is observed (21). Our preliminary observations by ASE–SEM revealed that, whereas the convex CP were always formed in all hair fibers, the convex SP varied among different hair fibers. Therefore, using ASE–SEM, we

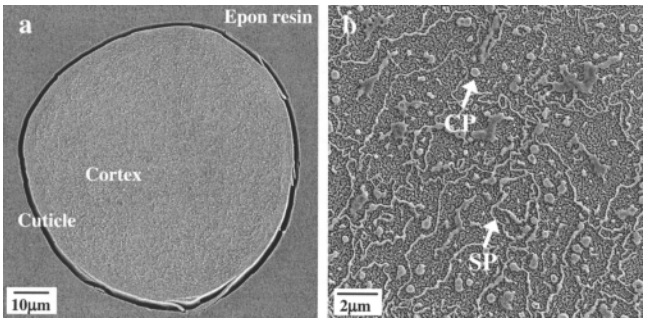

FIG. 1. Representative argon sputter etching–scanning electron microscopy (ASE–SEM) images of a transversely polished hair plane embedded in Epon resin. (a) A whole hair plane image, where cuticle, cortex, and Epon resin are observed. (b) A magnified image for part of the cortex in image a, where a circular pattern (CP) and a stitch pattern (SP) are observed.

characterized the variation of the convex SP in hair fibers derived from 27 Japanese females. ASE–SEM images are shown in Figure 2 for Japanese female No. 2, in Figure 3 for No. 13, and in Figure 4 for No. 24. In all hair planes of No. 2, ASE–SEM images consisted of both convex CP and SP (Fig. 2), which resembled those observed previously in virgin hair fibers (21). Convex CP but not convex SP were observed in ASE–SEM images in all hair planes of No. 13 (Fig. 3), which were almost the same as those in hair fibers delipidized by incubation with chloroform/methanol/water prior to the ASE procedures (21). In the ASE–SEM images of hair fibers of No. 24, convex CP were commonly observed in all hair fibers, but the convex SP in the hair plane varied in membranous morphology as well as in their 3D structures among the five fibers observed (Fig. 4). In some fibers, convex SP were observed sparsely over the hair planes (Fig. 4a,c), whereas in others, convex SP were observed densely and homogeneously (Fig. 4b,d,e). These observations demonstrate that there are individual variations and differences in the convex SP even among fibers derived from the same person.

Based on the variation in convex SP, ASE–SEM images were classified into Types 0 to 4 and scored with a 0 to 4 grading system, respectively, as shown in Table 1. Representative ASE–SEM images corresponding to Types 0 through 4 are depicted in Figure 5. For Type 0 (score = 0) convex SP characteristically were not detectable (Fig. 5a). All ASE–SEM images in Figure 3 were classified as Type 0. Type 1 (score = 1) was assigned according to a characteristic in which a thin convex SP was seen sparsely (Fig. 5b). The ASE–SEM image for Figure 4c was classified as Type 1. For Type 2 (score = 2) a thin convex SP was observed over most of the plane (Fig. 5c). The ASE–SEM image for Figure 4a was classified as Type 2. Type 3 (score = 3) was assigned by a characteristic in which both thin and thick convex SP were observed over all of the plane (Fig. 5d). The ASE–SEM image for Figure 4e was classified as Type 3. Type 4 (score = 4) was assigned according to a characteristic in which a thick convex SP was observed all over the plane (Fig. 5e). The ASE–SEM images for all fibers in Figure 2 and for Figure 4b and 4d were classified as Type 4. The types of ASE–SEM images and their scores for 27 Japanese females according to this classification are listed in Table 2. Since the averages and the SE of the scores reflect inter- and intraindividual variation, respectively, their values (as shown in Table 2) allow us to evaluate the differences in ASE–SEM images among females, as well as among fibers from each female.

Relationship between ASE–SEM images and lipid composition. Levels of SQ, WEs, TGs, FFAs, CH, CER/PDS, and CS in 27 Japanese females were determined using the proximal root regions, which were close to the observation sites of the ASE–SEM images for each female, by TLC–FID, GC–MS and LC–MS (18), as listed in Table 3. The lipid analyses demonstrated that although the lipid composition varied among the females tested, the levels of exogenous lipids (SQ, WEs, TGs, and FFAs) were in general greater than endogenous ones (CH, CER/PDS, and CS); and FFAs and WEs were predominantly present. These findings are in agreement with our previous re-

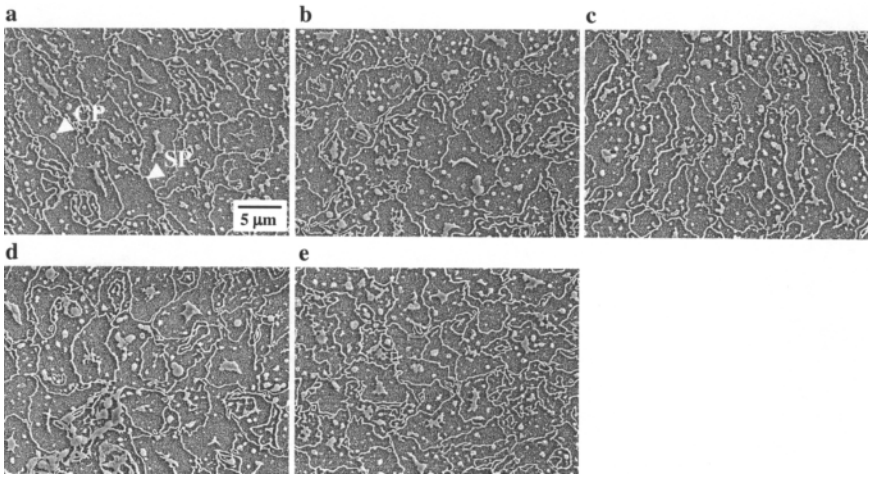

FIG. 2. ASE–SEM images in Japanese female No. 2. The images a–e, which include a CP and an SP, were obtained from five different hair fibers from the same scalp. For abbreviations see Figure 1.

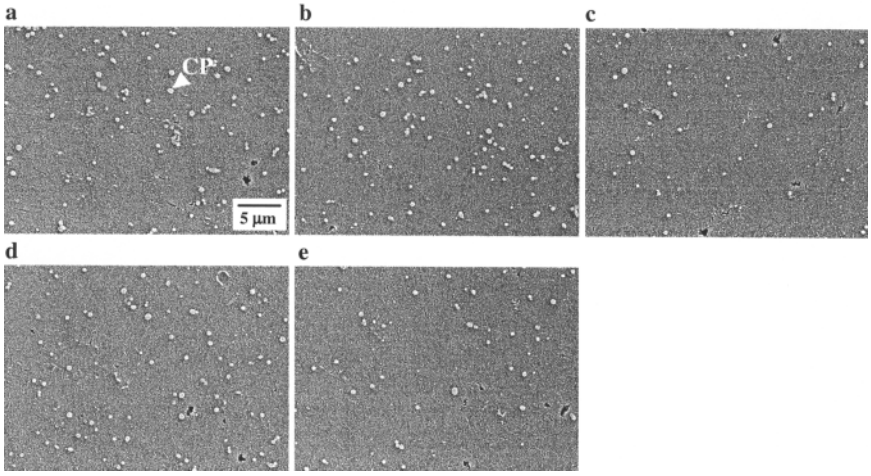

FIG. 3. ASE–SEM images in Japanese female No. 13. The images a–e, which include a CP, were obtained from five different hair fibers from the same scalp. For abbreviations see Figure 1.

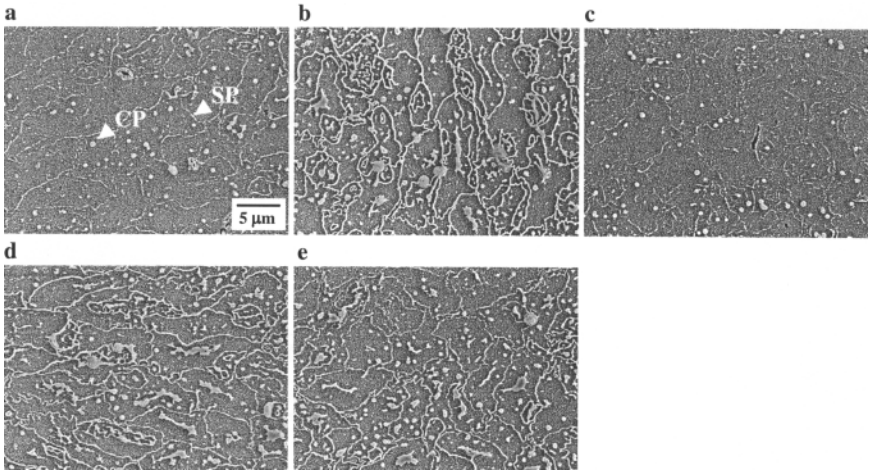

FIG. 4. ASE–SEM images in Japanese female No. 24. The images a–e, which include a CP and an SP, were obtained from five different hair fibers from the same scalp. For abbreviations see Figure 1.

ports (18,20). Table 4 shows the correlation coefficients between the average scores of the ASE–SEM images (Table 2) and the levels of hair lipids (Table 3) for 27 Japanese females. The average scores had significant positive correlations with the levels of WEs (R = 0.846), FFAs (R = 0.813), SQ (R = 0.530) and TGs (R = 0.424), and significant negative correlations with the levels of CH (R = −0.892) and CER/PDS (R = −0.688). The level of CS did not correlate with the average scores. A relationship between the average scores and the levels of WEs, which had the highest positive correlation, is shown in Figure 6. This correlation indicates that hair fibers with higher average scores in the ASE–SEM images contain higher levels of WEs as well as other exogenous FFAs, SQ, and TGs.

Characterization of ASE–SEM images in relation to aging. Individual hair fibers cut just at the proximal root end were collected from 380 females, consisting of 153 Japanese (Mongoloid), 169 Germans and 58 Americans (Caucasoid), and the transverse hair planes at the 1-cm region from the proximal root end were then observed using ASE–SEM. Table 5 shows the types of ASE–SEM images obtained from the hair fibers of 153 Japanese females. No females with Type 3 or Type 4 in their

TABLE 1
Scores and Features in Type 0 to Type 4[a] of Classification of ASE–SEM Images

	Score	Feature
Type 0	0	No convex SP is detectable.
Type 1	1	Thin convex SP is observed sparsely.
Type 2	2	Thin convex SP is observed mostly over the plane.
Type 3	3	Thin and thick convex SP is observed all over the plane.
Type 4	4	Thick convex SP is observed all over the plane.

[a]See Figure 5. ASM–SEM, argon sputter etching–scanning electron microscopy; SP, stitch pattern.

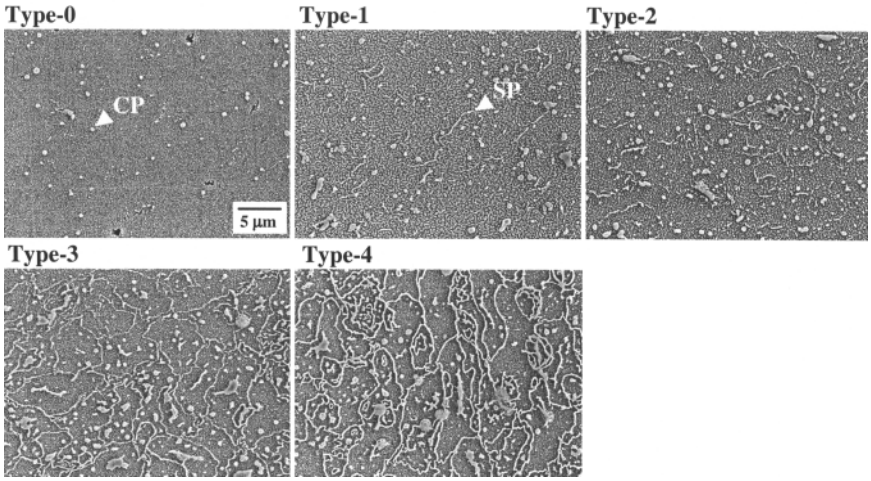

FIG. 5. Representative ASE–SEM images corresponding to Types 0–4. For abbreviation see Figure 1.

hair fibers were observed in the youngest group aged 0 to 9 yr. However, in the younger female group aged 10 to 29 yr, the percentages of Type 3 or Type 4 sharply increased, and in the older female group aged 50 to 69 yr, the percentages of Type 3 or Type 4 decreased (Table 5). Changes in the percentages of Type 0 or Type 1 according to aging were inversely related to those of Type 3 or Type 4. Table 6 shows the types of ASE–SEM images from the hair fibers of 169 German females. Changes in the types of ASE–SEM images for German females with aging are almost identical to those for Japanese females, although we had no individuals in the youngest German female group aged 0 to 9 yr. Thus, the percentages of females with Type 3 or Type 4 were greater in the younger group than in the older group, while those with Type 0 or Type 1 were greater in

TABLE 2
Scores of ASE–SEM Images in 27 Japanese Females

No.	Score[a]					Average	SE
1	4	4	4	4	4	4.0	0.0
2	4	4	4	4	4	4.0	0.0
3	4	3	3	3	1	2.8	0.6
4	0	0	1	0	0	0.2	0.2
5	0	1	1	0	0	0.4	0.3
6	4	2	3	3	2	2.8	0.4
7	0	0	0	1	0	0.2	0.2
8	2	2	2	2	2	2.0	0.0
9	1	2	2	2	1	1.6	0.3
10	1	2	2	2	2	1.8	0.2
11	1	2	1	2	3	1.8	0.4
12	2	1	1	1	2	1.4	0.3
13	0	0	0	0	0	0.0	0.0
14	2	1	3	2	1	1.6	0.4
15	2	3	1	0	1	1.2	0.6
16	3	2	3	2	4	2.8	0.4
17	2	3	4	4	4	3.4	0.4
18	4	4	4	4	4	4.0	0.0
19	0	0	0	0	0	0.0	0.0
20	0	0	1	1	0	0.4	0.3
21	0	0	0	1	0	0.2	0.2
22	4	4	4	4	1	3.4	0.7
23	1	1	1	4	4	2.2	0.8
24	2	4	1	4	3	2.8	0.7
25	4	4	4	4	4	4.0	0.0
26	4	4	4	4	4	4.0	0.0
27	1	1	0	1	1	0.8	0.2

[a]See Table 1 for abbreviation and explanation of score.

TABLE 3
Levels of Hair Lipids[a] in 27 Japanese Females

	Level (mg/g hair)						
No.	SQ	WEs	TGs	FFAs	CH	CER/PDS	CS
1	2.5	7.3	2.8	21.4	1.0	0.09	0.10
2	2.3	13.2	0.3	14.4	0.6	0.07	0.19
3	1.0	6.0	0.5	21.0	1.0	0.09	0.19
4	1.2	0.9	0.3	4.9	2.5	0.14	0.13
5	0.4	1.4	0.4	3.2	1.9	0.15	0.14
6	4.6	14.3	1.9	28.4	1.1	0.09	0.17
7	1.1	2.1	0.6	4.2	1.7	0.10	0.19
8	1.6	5.1	0.9	8.2	1.9	0.12	0.14
9	3.5	8.3	1.0	14.6	1.4	0.10	0.20
10	2.0	5.5	0.8	10.1	1.5	0.11	0.21
11	2.6	8.2	0.6	22.0	0.9	0.08	0.27
12	1.6	4.6	1.0	7.3	1.6	0.14	0.20
13	1.7	2.3	1.2	4.9	2.1	0.19	0.09
14	0.9	5.3	0.5	9.2	1.5	0.14	0.13
15	1.0	4.9	0.8	6.7	1.6	0.11	0.22
16	2.5	5.9	0.4	13.0	0.7	0.05	0.15
17	1.9	6.7	0.8	15.2	0.7	0.07	0.25
18	2.0	13.8	0.6	17.3	0.6	0.08	0.11
19	0.5	2.8	0.3	5.3	1.9	0.10	0.16
20	0.8	3.7	0.3	6.2	1.8	0.12	0.15
21	0.5	2.4	0.7	5.4	2.0	0.14	0.12
22	1.6	9.8	1.0	18.9	1.2	0.11	0.23
23	1.0	8.7	0.9	12.2	1.6	0.12	0.17
24	2.3	8.2	1.1	16.5	1.1	0.09	0.19
25	1.8	11.1	0.7	18.4	0.8	0.09	0.18
26	2.2	10.3	1.8	25.4	0.7	0.08	0.13
27	0.5	3.3	0.3	5.2	1.9	0.11	0.21

[a]SQ, squalene; WEs, wax esters; TGS, triglycerides; FFAs, free fatty acids; CH, cholesterol; CER/PDS, ceramide/N-palmitoyl-DL-dihydrosphingosine; CS, cholesterol sulfate.

TABLE 4
Correlation Coefficients Between the Average Scores of ASE–SEM Images and Levels of Hair Lipids in 27 Japanese Females

Hair lipid	Correlation coefficient with averaged scores	Hair lipid	Correlation coefficient with averaged scores
SQ	0.530**	CH	–0.892**
WEs	0.746**	CER/PDS	–0.688**
TGs	0.424*	CS	0.121
FFAs	0.813**		

**$P < 0.01$; *$P < 0.05$. For abbreviations see Tables 1 and 3.

TABLE 5
Types[a] of ASE–SEM Images of Hair Fibers from 153 Japanese (Mongoloid) Females

	Number of females (%)					
Age	Type 0	1	2	3	4	Total
0–9	11 (61)	6 (33)	1 (6)	0 (0)	0 (0)	18
10–19	2 (12)	2 (12)	3 (18)	3 (18)	7 (41)	17
20–29	3 (10)	4 (13)	7 (23)	4 (13)	12 (40)	30
30–39	7 (17)	4 (10)	10 (24)	12 (29)	9 (21)	42
40–49	6 (21)	6 (21)	5 (18)	6 (21)	5 (18)	28
50–59	4 (30)	2 (17)	2 (17)	1 (8)	3 (25)	12
60–69	3 (50)	0 (0)	2 (33)	1 (17)	0 (0)	6
Total	36 (23)	24 (16)	30 (20)	27 (18)	36 (23)	153

[a]See Figure 5. For abbreviation see Table 1.

the older group than in the younger group (Table 6). There was a marked increase in the percentages of German females with Type 0 or Type 1 with aging, probably because only German females included the oldest group, aged 70 to 79 yr (Tables 5, 6). Table 7 shows the types of ASE–SEM images from the hair fibers of 58 American females. Changes in the types of ASE–SEM images for American females with aging (Table 7) were almost identical to those for Japanese and German females, although we had no individuals in the youngest group aged 0 to 9 yr or in the oldest group aged 70 to 79 yr.

The average scores for the hair fibers from all 380 females tested are shown in Table 8. Although the ASE–SEM images of the youngest female group aged 0 to 9 yr were taken only of Japanese, the average score of the ASE–SEM images in the youngest female group was found to be the lowest. Common characteristics associated with age-related changes were observed in all races (Mongoloid and Caucasoid); younger females aged 10 to 29 yr had higher scores and older females aged 50 to 69 yr showed lower scores (Table 8). In the oldest females aged 70 to 79 yr, who were only Germans, a marked decrease in the average score was observed dependent on aging.

DISCUSSION

Our previous study, which used the proximal root regions of hair fibers collected from 44 different Japanese females (1 to 81 yr of age), revealed that the lipid composition of hair in females varies from person to person as well as with age (20). The predominant variation was a negative correlation between exogenous lipids (SQ, WEs, TGs, and FFAs) and endogenous

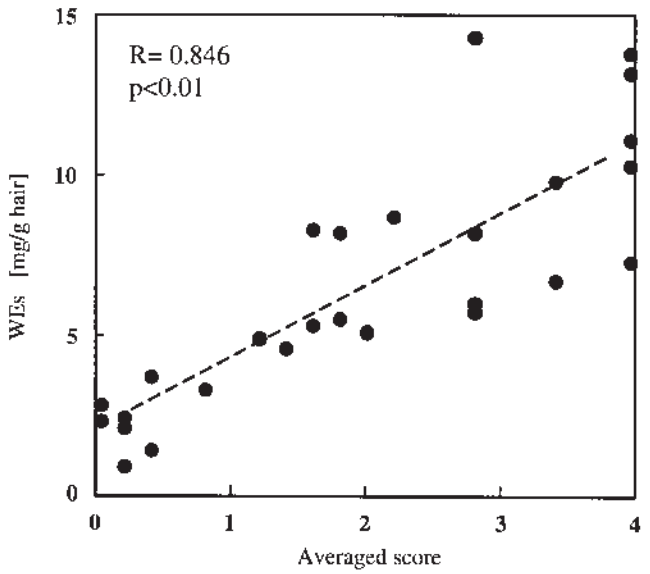

FIG. 6. Representative relationship between average scores of ASE–SEM images and levels of wax esters (WEs). Each point corresponds to the score and the level for each individual. For other abbreviation see Figure 1.

TABLE 6
Types[a] of ASE–SEM Images of Hair Fibers from 169 German (Caucasoid) Females

	Number of females (%)					
Age	Type 0	1	2	3	4	Total
10–19	0 (0)	0 (0)	2 (50)	2 (50)	0 (0)	4
20–29	2 (6)	5 (14)	6 (17)	8 (22)	15 (42)	36
30–39	6 (10)	8 (14)	15 (26)	17 (29)	12 (21)	58
40–49	6 (21)	5 (17)	7 (24)	6 (21)	5 (17)	29
50–59	4 (27)	2 (13)	2 (13)	3 (20)	4 (27)	15
60–69	5 (29)	5 (29)	6 (35)	1 (6)	0 (0)	17
70–79	5 (50)	3 (30)	1 (10)	1 (10)	0 (0)	10
Total	28 (17)	28 (17)	39 (23)	38 (22)	36 (29)	169

[a]See Figure 5. For abbreviation see Table 1.

TABLE 7
Types[a] of ASE–SEM Images of Hair Fibers from 58 American (Caucasoid) Females

	Number of females (%)					
Age	Type 0	1	2	3	4	Total
10–19	1 (14)	2 (29)	0 (0)	2 (29)	2 (29)	7
20–29	1 (13)	1 (13)	1 (13)	8 (25)	3 (38)	8
30–39	6 (22)	2 (7)	6 (22)	8 (30)	5 (19)	27
40–49	2 (25)	1 (13)	0 (0)	2 (25)	3 (38)	8
50–59	1 (25)	0 (0)	1 (25)	2 (50)	0 (0)	4
60–69	2 (50)	1 (25)	1 (25)	0 (0)	0 (0)	4
Total	13 (22)	7 (12)	9 (16)	16 (28)	13 (22)	58

[a]See Figure 5. For abbreviation see Table 1.

TABLE 8
Average Scores[a] of ASE–SEM Images of Hair Fibers from All Females Observed

	Averaged score of ASE–SEM images			
Age	Japanese (n = 153)	German (n = 169)	American (n = 58)	All (n = 380)
0–9	0.44	—	—	0.44
10–19	2.65	2.50	2.29	2.54
20–29	2.60	2.81	2.63	2.70
30–39	2.29	2.36	2.15	2.29
40–49	1.93	1.97	2.38	2.00
50–59	1.75	2.07	2.00	1.94
60–69	1.17	1.18	0.75	1.11
70–79	0.80	0.80		
Total	2.02	2.15	2.16	2.10

[a]See Table 1. For abbreviation see Table 1.

lipids (CH and CERs), which suggests that endogenous lipids might serve as a barrier against the penetration of sebum-derived exogenous lipids. In addition, age-related changes in the composition of exogenous hair lipids are found, which seem to reflect an increased sebum secretion during adolescence (20). On the other hand, we recently established the ASE–SEM method to visualize hair lipids at the CMC in hair fibers (21). In ASE–SEM images, the convex SP in a transversely polished hair plane would be considered an indicator of the localization of hair lipids, based on the fact that whereas virgin hair fibers have convex SP in their polished hair planes, solvent-treated hair fibers lack convex SP (21).

The present study reveals that the convex SP in ASE–SEM images among females includes not only inter- but also intraindividual variation among hair fibers. Since a convex SP is associated with hair lipids, these variations reflect differences in hair lipids among females as well as among fibers derived from each female. We used the interindividual variation among females to determine which lipids predominantly contribute to the convex SP. According to characteristics in frequency and

strength of the convex SP, ASE–SEM images were classified into Type 0 through 4, and scored as 0–4, respectively, as shown in Table 1 and Figure 5. When relationships between the average scores obtained from ASE–SEM images and the measured levels of hair lipids were evaluated for 27 Japanese females, we found significant positive correlations between the scores and the levels of WEs, FFAs, SQ, and TGs (as listed in Table 4). This indicates that a convex SP is attributable to the levels of exogenous lipids such as WEs, FFAs, SQ, and TGs. The contribution of exogenous lipids to the formation of convex SP is corroborated by our previous study, which showed that, although delipidized hair fibers had no convex SP in ASE–SEM images, treatment with FA and wax esters induced the reappearance of convex SP in the hair plane (21). We also found significant negative correlations between the average scores and the levels of endogenous lipids such as CH and CER/PDS, as listed in Table 4, in which higher levels of endogenous lipids in hair fibers correlated with fewer convex SP in ASE–SEM images. Since the higher levels of endogenous lipids are paralleled by lower levels of exogenous lipids that have penetrated into the hair fibers, these negative correlations may support the possibility that a convex SP is mainly composed of exogenous lipids that have penetrated from the surface against the possible barrier function regulated by endogenous lipids.

The present study also suggests the notion of an internalized site for exogenous lipids consisting of sebum excreted at the hair follicle and diffused within a hair fiber. Although the existence of exogenous lipids within hair fibers is well known (7–9,11,13,20), such an internalized site has not been hitherto reported. However, our study indicates that exogenous lipids, such as SQ, WEs, TGs, and FFAs, are mainly associated with the convex formation of SP at the CMC in ASE–SEM images, which suggests in turn that the internalization of exogenous lipids within hair fibers mainly occurs at the CMC.

Furthermore, the intraindividual variation of the convex SP suggests another implication about the heterogeneous features of hair fibers growing on the scalp. Since levels of exogenous lipids had been previously determined using bundles of hair and by conventional analytical methods (7–13,18,20), their variation along a single hair fiber on the scalp had not been detectable. However, the observed intraindividual variation in the convex SP seen in this study suggests that ASE–SEM can roughly measure the variation in levels of exogenous lipids along hair fibers derived from the scalp. Thus, because the contents of exogenous lipids seem to be closely related to physicochemical properties such as hydrophobicity, water holding, and elasticity of hair fibers, the determination of the variation in the levels of exogenous lipids by ASE–SEM would be helpful when considering the properties of scalp hair fibers. If a female has variations in the levels of exogenous lipids among her hair fibers, her scalp hair can be considered physicochemically uneven, which might result in heterogeneous properties, including its texture and appearance.

Taken together, the advantages of the ASE–SEM method can be summarized as follows: (i) The interindividual variation can be useful for characterizing exogenous lipids among individuals, which may contribute to understanding different hair fibers in relation to age, sex, and race, (ii) the intraindividual variation also can be useful for characterizing exogenous lipids among fibers derived from an individual, which may allow us to clarify physicochemical properties of scalp hair fibers. In this study, results from 380 hair fibers from 380 females revealed that levels of exogenous lipids, which reflect convex SP, changed in relation to aging. The youngest females aged 0 to 9 yr (only Japanese) have lower levels of exogenous lipids, whereas hair from younger females aged 10 to 29 yr tends to contain higher levels of exogenous lipids. Further, older females aged 50 to 69 yr, and in particular, the oldest females aged 70 to 79 yr (only Germans), have lower levels of exogenous lipids. The age-related changes in the levels of exogenous lipids agree with our previous study that described hair lipid composition and its variation in Japanese females aged 1 to 81 yr (20). These changes seem to be similar regardless of racial origin tested. Since sebaceous excretion is known to increase during adolescence in females and then decrease gradually after menopause (22,23), the lower levels of exogenous lipids may be ascribed to a reduced excretion of sebum in the youngest or oldest females.

Changes in hair with aging have been reported by other groups with respect to the level of cholesterol (19), water content (16), diameter, and physical properties (24). As for the physical properties, Naruse and Fujita examined hair fibers collected from 81 Japanese females aged 2 to 91 yr with stress-strain curves; the physical parameters, such as breaking strength, breaking extension, and breaking energy, showed the highest values in 15–20 yr-old women and then gradually decreased with aging (24). These changes in the physical parameters of female hair are similar to those of the levels of exogenous lipids in this study. This suggests that age-related changes in exogenous lipids may be associated with changes in physical parameters of female hair, such as hydrophobicity, water holding and elasticity, which affect their texture with respect to moisture content, smoothness, and flexibility.

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Lower Weight Gain and Higher Expression and Blood Levels of Adiponectin in Rats Fed Medium-Chain TAG Compared with Long-Chain TAG

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ABSTRACT: Previous studies demonstrated that, compared with long-chain TAG (LCT), dietary medium-chain TAG (MCT) could improve glucose tolerance in rats and humans. It has been well established that adiponectin acts to increase insulin sensitivity. The effects of dietary MCT on adiponectin serum concentration and mRNA levels in adipose tissue were studied in rats. Male Sprague-Dawley rats were fed a diet containing 20% MCT or LCT for 8 wk. After 6 wk of dietary treatment, an oral glucose tolerance test was performed. Rats fed the MCT diet had less body fat accumulation than those fed the LCT diet ($P < 0.01$). The cell diameter of the perirenal adipose tissue, one of the abdominal adipose tissues, was smaller ($P < 0.01$) in the MCT diet group. The serum adiponectin concentration was higher ($P < 0.01$) in the MCT diet group than in the LCT diet group. The adiponectin content in the perirenal adipose tissue was higher ($P < 0.01$) in the MCT diet group. The MCT-fed group had a higher adiponectin mRNA level in their perirenal adipose tissue ($P < 0.05$). The increase of the plasma glucose concentration after glucose administration (area under the curve) was smaller ($P < 0.01$) in the MCT diet group than in the LCT diet group. These findings suggest that dietary MCT, compared with LCT, results in a higher serum adiponectin level with transcriptional activation of the adiponectin gene in rats. We speculate that improved glucose tolerance in rats fed an MCT diet may be, at least in part, ascribed to this higher serum adiponectin level.

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Medium-chain TAG (MCT) are edible oils consisting of medium-chain FA. The digestion, absorption, and metabolism of MCT are markedly different from those of long-chain TAG (LCT) (1). Animal and human studies have provided evidence that MCT impact not only lipid metabolism (1–4) but also glucose metabolism (5,6). Han *et al.* (5) found improved glucose tolerance and insulin sensitivity in MCT-fed rats, compared with LCT. Eckel *et al.* (6) reported that an MCT diet, compared with an LCT diet, increased insulin sensitivity in both diabetic patients and nondiabetic subjects.

Adiponectin (also known as ACRP30, apM1, and AdipoQ) is one of the adipocytokines secreted by mature adipocytes that circulate in high concentrations in the blood (7). It is well established that adiponectin acts to increase insulin sensitivity (8). A high-fat diet increased the adipocyte size of the adipose tissue, and at the same time decreased the adiponectin mRNA levels in mice (9). These studies indicate that the suppression of adipocyte hypertrophy might lead to a higher plasma adiponectin level, resulting in protection against insulin resistance (10).

Previous studies showed that the cell size of adipose tissue was significantly smaller in animals fed MCT than in those fed LCT (3,11,12). We hypothesized that dietary MCT might increase serum adiponectin concentration by transcriptional activation of the adiponectin gene in adipose tissue, resulting in improved glucose tolerance. Therefore, we investigated the effects of dietary MCT on the serum adiponectin concentration and adiponectin mRNA level in adipose tissue of rats.

MATERIALS AND METHODS

Animals and diets. All animals were treated in accordance with the guidelines for the care and use of laboratory animals (Notification of the Prime Minister's Office in Japan). The experimental plan was approved by the Laboratory Animal Care Committee of the Research Laboratory, Nisshin Oillio Group. Male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) were individually housed in stainless steel wire cages and allowed free access to sterilized water. The temperature of the animal room was set at 23 ± 1°C, with RH of 50 ± 5% and illumination from 0800 to 2000 h. The animals were also allowed free access to a commercial stock diet before the experiment.

Eight-week-old rats were randomized into two groups. Each group of rats ($n = 10$ or 9) was allowed free access to the experimental diet containing 20% of one of the test oils for 8 wk. The experimental diets contained the following ingredients (g/kg): cornstarch, 249.955; casein, 200; test oil, 200; soybean oil, 30; AIN-93G mineral mix (13), 35; AIN-93 vitamin mix (13), 10; cellulose, 269.5; L-cystine, 3; choline bitartrate, 2.5; and TBHQ, 0.045. The soybean oil, MCT, and rapeseed oil were purchased commercially (Nisshin Oillio Group, Tokyo, Japan). The rapeseed oil was used as the LCT. The FA compositions of the test

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Abbreviations: AUC, area under the curve; CBP, cAMP response element binding-protein binding protein; CREB, cAMP response element binding-protein; LCT, long-chain TAG; LRH-1, liver receptor homolog-1; MCT, medium-chain TAG; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, peroxisome proliferator-activated receptor-responsive element; RXR, retinoid X receptor; RT, reverse transcription; TNF-α, tumor necrosis factor α.

TABLE 1
FA Composition of Test Oils

	LCT	MCT
8:0[a]	—	75.5
10:0	—	24.5
16:0	4.6	—
18:0	2.2	—
18:1	62.5	—
18:2	20.3	—
18:3	7.2	—
20:0	0.7	—
20:1	1.3	—
Others	1.2	—

[a]Number of carbon atoms: number of double bonds. LCT, long-chain TAG; MCT, medium-chain TAG.

oils (Table 1) were determined by GLC (6890 series; Agilent Technologies, Palo Alto, CA) with a capillary column (SP2340; Supelco, Bellefonte, PA) (4). The digestible energy of the two diets was calculated as the gross energy fed minus the energy in feces. The energies of the diets and feces were determined using a bomb calorimeter (CA-4P, Shimadzu, Kyoto, Japan).

At the end of the feeding period, the rats were killed by decapitation after 6 h of food deprivation. In a separate experiment, we confirmed that rats were not hungry after 6 h of food deprivation. Blood was collected to obtain serum, which was stored at −80°C until use. The abdominal adipose tissues (perirenal, epididymal, and mesenteric) were carefully removed using scissors and weighed. Carcass samples were obtained by removing the abdominal adipose tissues and liver and were stored at –80°C until analyses.

Glucose tolerance test. After 6 wk of dietary treatment, an oral glucose tolerance test was performed. The animals were food-deprived for 6 h. Blood samples were obtained from the tail vein using heparinized syringes at 0, 30, 60, 90, 120, and 180 min after the oral administration of 2 g glucose/kg body wt. The area under the curve (AUC) for glucose and insulin was determined using the trapezoidal rule (14).

Analyses. The carcass fat content was analyzed by a method reported previously (15). The adipose cell size was determined in osmium-fixed cells as described by Hirsch and Gallian (16). Briefly, adipose tissue samples were placed in plastic vials containing osmium tetroxide and collidine for immediate fixation. Postfixation, connective tissue and debris were removed *via* filtering through 250 and 25 μm nylon mesh screens with saline. The cell size was determined using a laser scattering particle-size analyzer (LA-500, Horiba, Kyoto, Japan). The fat cell number was calculated using the following formula: number of cells/g = average cell size (pL) × 0.95 (ng lipid/1 pL) × g/10^9 ng (17). This product was then multiplied by the number of grams of tissue to get the fat cell number.

The adiponectin levels in the serum and adipose tissue were measured by an ELISA kit (Otsuka Phamaceutical, Tokyo, Japan) according to the manufacturer's instructions. The adiponectin in the adipose tissue was extracted with 10 mmol/L HEPES containing 1 mmol/L EDTA and 1% NP-40. The plasma glucose and insulin concentrations were determined by using a colorimetric assay kit (Wako Pure Chemical, Osaka, Japan) and ELISA kit (Shibayagi, Shibukawa, Japan), respectively.

The mRNA levels of adiponectin, retinoid X receptor (RXR), peroxisome proliferator-activated receptor γ (PPARγ), liver receptor homolog-1 (LRH-1), tumor necrosis factor α (TNF-α), and cAMP response element binding-protein (CREB) binding protein (CBP) in the perirenal adipose tissue were measured by quantitative real-time PCR (LightCycler PCR; Roche, Basel, Switzerland). The total RNA from the adipose tissue (80–100 mg) was extracted from each adipose tissue using RNeasy Lipid Tissue Mini (Qiagen, Hilden, Germany). Reverse transcription (RT) (1 μg of total RNA) was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The following sense and antisense primers were used (GenBank accession numbers are in parentheses): adiponectin (AY033885), nucleotides 130–148 and 250–268; RXR (L06482), nucleotides 908–927 and 1037–1056; PPARγ (AF156665), nucleotides 1269–1288 and 1417–1436; LRH-1 (NM 021742), nucleotides 1350–1369 and 1478–1497; CBP (AY462245), nucleotides 5123–5142 and 5222–5241; TNF-α (X66539), nucleotides 150–168 and 291–309; β-actin (NM 031144), nucleotides 939–958 and 1095–1114. The data were analyzed according to the comparative cycle threshold method and were normalized by the β-actin expression in each sample.

Statistical analysis. Data were expressed as mean ± SEM. The results were analyzed by the *F*-test for equality of variance, and significant differences between the diet groups were determined by the Student's or Welch's *t*-test. Differences with a value of $P < 0.05$ were considered significant. A statistical software package (Toukei version 2; Esumi, Tokyo, Japan) was used for all of the statistical analyses.

RESULTS

Body weight, food intake, and body fat mass. The body weight gain was significantly lower in the rats fed the MCT diet than in those fed the LCT diet (Table 2). There was no significant difference in food and digestible energy intake between the two diet groups. The weight of the abdominal adipose tissue was significantly lower in the MCT diet group than in the LCT diet group. The carcass fat content was also significantly lower in the MCT-fed rats.

Cell size, cell number, and adiponectin content of adipose tissues. The weight of the perirenal adipose tissue was significantly lower in the rats fed the MCT diet than in those fed the LCT diet (Table 3). The cell diameter of the perirenal adipose tissue in the rats fed the MCT diet was smaller than those fed the LCT diet. However, the cell numbers of the two groups were the same. The adiponectin content (expressed as per gram of adipose tissue and per 10^6 cells) in the perirenal tissue was significantly higher in the MCT diet group than in the LCT diet group.

Serum adiponectin, leptin, glucose, and insulin. The serum adiponectin concentration was significantly higher in the rats fed the MCT diet than in those fed the LCT one (2.6 ± 0.2 vs.

TABLE 2
Body Weight Gain, Food Intake, and Body Fat in Rats Fed LCT or MCT Diets[a]

	Diet	
	LCT	MCT
Initial body weight (g)	298 ± 2	298 ± 2
Body weight gain (g/8 wk)	153 ± 7	127 ± 5**
Food intake (g/d)	20.8 ± 0.4	19.9 ± 0.4
Digestible energy intake (kJ/d)	368 ± 7	349 ± 7
Abdominal adipose tissues (g)	29.9 ± 2.0	21.4 ± 1.5**
Carcass fat (g)	48.8 ± 2.8	37.7 ± 2.5**

[a]Values are means ± SEM, n = 10 (LCT) or 9 (MCT). Asterisks indicate different from LCT diet: **P < 0.01. For abbreviations see Table 1.

TABLE 3
Weight, Mean Cell Diameter, Cell Number, and Adiponectin Content of Perirenal Adipose Tissue in Rats Fed LCT or MCT Diets[a]

	Diet	
	LCT	MCT
Weight (g)	12.4 ± 0.8	9.3 ± 0.6**
Mean cell diameter (μm)	119 ± 2	109 ± 2**
Cell number (10^6 cell/perirenal fat pad)	14.7 ± 0.7	13.8 ± 1.4
Adiponectin content (μg/g)	3.5 ± 0.2	8.2 ± 0.9**
(μg/10^6 cells)	3.0 ± 0.2	5.4 ± 0.6**

[a]Values are means ± SEM, n = 10 (LCT) or 9 (MCT). Asterisks indicate different from LCT diet: **P < 0.01. For abbreviations see Table 1.

5.1 ± 0.5 mg/L, Table 4). There was no significant difference in the serum leptin concentration between the two diet groups. The rats fed the MCT diet had a significantly lower serum glucose concentration than those fed the LCT diet. The type of dietary fat did not affect the insulin concentration.

Adiponectin, RXR, PPARγ, LRH-1, TNF-α, and CBP mRNA in adipose tissue. There was no significant difference in β-actin expression between the LCT and MCT groups (100 ± 7 vs. 98 ± 8% of LCT diet group). The adiponectin mRNA level in the perirenal adipose tissue was significantly higher in the rats fed the MCT diet than in those fed the LCT diet (129 ± 9 vs. 100 ± 8% of LCT diet group, Table 5). The RXR and PPARγ mRNA levels were also significantly higher in the rats fed the MCT diet. The rats fed the MCT diet tended (P = 0.06) to have a higher LRH-1 mRNA level than those fed the LCT diet. The TNF-α and CBP mRNA levels of perirenal adipose tissue were found to be similar between the two diet groups.

Oral glucose tolerance test. The plasma glucose concentration before glucose administration was not significantly different between the two diet groups (Fig. 1A). The plasma glucose

TABLE 4
Serum Glucose, Insulin, and Adiponectin Concentration in Rats Fed LCT or MCT Diets[a]

	Diet	
	LCT	MCT
Adiponectin (mg/L)	2.6 ± 0.2	5.1 ± 0.5**
Leptin (μg/L)	6.2 ± 0.5	5.5 ± 0.5
Glucose (mmol/L)	9.0 ± 0.2	8.4 ± 0.1*
Insulin (pmol/L)	0.18 ± 0.03	0.21 ± 0.04

[a]Values are means ± SEM, n = 10 (LCT) or 9 (MCT). Asterisks indicate different from LCT diet: *P < 0.05; **P < 0.01. For abbreviations see Table 1.

TABLE 5
Relative mRNA Levels of Perirenal Adipose Tissue in Rats Fed LCT or MCT Diets[a]

	Diet	
	LCT	MCT
	(% of LCT diet group)	
Adiponectin	100 ± 8	129 ± 9*
RXR	100 ± 9	132 ± 9*
PPARγ	100 ± 3	138 ± 13*
LRH-1	100 ± 6	139 ± 18
TNF-α	100 ± 9	108 ± 11
CBP	100 ± 8	84 ± 9

[a]Values are means ± SEM, n = 10 (LCT) or 9 (MCT). Asterisks indicate different from LCT diet: *P < 0.05. RXR, retinoid X receptor; PPARγ, peroxisome proliferator-activated receptor γ; LRH-1, liver receptor homolog-1; TNF-α, tumor necrosis factor α; CBP, cAMP response element binding-protein (CREB) binding protein. For other abbreviations see Table 1.

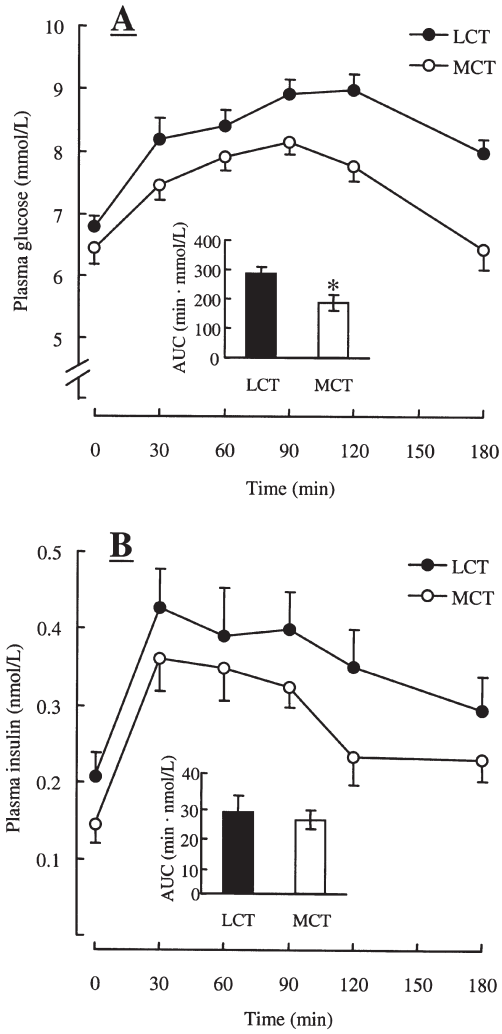

FIG. 1. Plasma glucose (A) and insulin (B) responses after oral administration (2 g glucose/kg body wt) in rats fed long-chain TAG (LCT) or medium-chain TAG (MCT) for 6 wk. The areas under the curve (AUC) during the 180 min period after oral glucose administration were determined using the trapezoidal rule. Values are means ± SEM, n = 10 (LCT) or 9 (MCT).

concentration gradually increased after glucose administration, and the levels reached peaks after 90 min in the MCT diet group and 120 min in the LCT diet group. For 180 min after glucose administration, the plasma glucose levels appeared to be lower at all time points in the MCT diet group than in the LCT diet group. The increase in the plasma glucose concentration during the 180 min period after oral glucose administration (AUC) was significantly lower in the rats fed the MCT diet than in those fed the LCT one.

There was no significant difference in the plasma insulin level before glucose administration between the two diet groups (Fig. 1B). The plasma insulin levels of the two groups increased immediately (30 min), and then decreased gradually. The plasma insulin levels also appeared to be lower at all time points in the MCT diet group than in the LCT diet group. However, the increase of the plasma insulin concentration during the 180 min period after glucose administration (AUC) was not significantly different between the two diet groups.

DISCUSSION

Here, we demonstrated that rats fed a diet containing MCT had a higher serum adiponectin concentration than rats fed a diet containing LCT. Adiponectin is a protein exclusively secreted by adipocytes (7). We also observed that rats fed the MCT diet had a higher adiponectin content and mRNA level in their perirenal adipose tissue than those fed the LCT diet. These results indicate that the intake of MCT, compared with LCT, might promote adiponectin production by an increase of the mRNA expression in adipose tissue, resulting in the elevation of the plasma adiponectin concentration. To our knowledge, this is the first time that the effect of MCT on the serum concentration and mRNA level of adiponectin has been reported. The link between adiponectin and visceral adiposity and insulin resistance has been discussed (8). Therefore, we focused on abdominal adipose tissues in the present study.

In this study, the effects on adiponectin expression and blood levels of 8-wk consumption of LCT and MCT diets were compared. We did not have data on adiponectin expression and blood levels before intake of the test diets. The adiponectin levels in rats fed a standard chow diet (Labo MR Stock; Nosan Corporation, Yokohama, Japan) at the age of 8 and 16 wk were 3.9 ± 0.4 and 2.9 ± 0.4 mg/L, respectively (obtained from a separate experiment). Much more research is needed to clarify the changes in adiponectin expression and blood levels of adiponectin by intake of a MCT diet.

Results from animal and human studies show that adiponectin increases insulin sensitivity (8,9,18–25). Low adiponectin levels have been strongly implicated in the development of insulin resistance in mouse models of both obesity and lipoatrophy (9). Adiponectin knockout mice exhibited severe diet-induced insulin resistance with reduced insulin signaling activity in muscle (19). The administration of adiponectin improves glucose metabolism and insulin sensitivity in animal models of obesity and insulin resistance (9,20,21). In humans, circulating adiponectin concentrations are significantly lower in cases of obesity, type 2 diabetes, and insulin resistance (22). Lindsay *et al.* (23) demonstrated that plasma adiponectin levels were lower in Pima Indians, a unique cohort with a high prevalence of obesity, with diabetes. They also demonstrated that the plasma levels of adiponectin are strongly correlated with insulin sensitivity as evaluated by the glucose disposal rate (24). These studies show that adiponectin plays a key role in the prevention of diabetes mellitus (25).

The suppression of adipocyte hypertrophy leads to a higher plasma adiponectin level (10). In the present study, the cell size of the perirenal adipose tissue in the rats fed the MCT diet was smaller than that in rats fed the LCT diet. It is likely that the higher expression and serum level of adiponectin in the MCT group resulted from the suppression of adipocyte hypertrophy, rather than being a result of the MCT *per se*. On the other hand, PPARγ ligands prevent adipocyte hypertrophy and increase the expression and plasma concentration of adiponectin (26,27). Unsaturated FA are natural ligands for PPARγ. However, saturated FA, including medium-chain FA, are poor PPARγ ligands compared with unsaturated FA (28). Hence, it may be difficult to explain the transcriptional activation of the adiponectin gene in the MCT diet group by the ligand action of medium-chain FA.

MCT are metabolized differently from LCT (1). On one hand, ingested MCT are more easily degraded to FA and glycerol by pancreatic lipase compared with LCT and are absorbed directly into the portal circulation and transported to the liver for rapid oxidation. On the other hand, absorbed LCT flow into the veins *via* lymphatic vessels and are transported to peripheral tissues, such as adipose tissues and muscle tissues. Therefore, the fast rate of oxidation of MCT increases energy expenditure and results in less body fat accumulation (13,29,30). We have reported larger diet-induced thermogenesis in rats fed MCT than in those fed LCT (30). Diet-induced thermogenesis occurs mainly in brown adipose tissue regulated by the sympathetic nervous system (31,32). Thus, the fast oxidation rate of MCT might relate to the activity of the sympathetic nervous system and uncoupling protein. In this study, we also observed less body fat accumulation in the MCT diet group than in the LCT diet group. The mass of adipose tissue is dependent on the size and number of its adipocytes (33). Previous studies reported that the adipocyte size (but not adipocyte number) was smaller in the MCT-fed than in the LCT-fed rats, using rats with >250 g body weight (3,11,12). In the present study using 8-wk-old rats, we also observed a smaller adipocyte size in the MCT diet group, whereas the adipocyte number was not affected by dietary fat. However, Hashim and Tantibhedyangkul (33) investigated the effects of feeding a MCT diet on the development of adipose tissue in postweanling rats. They reported that the MCT-fed rats had a smaller size and number of adipocytes than the LCT-fed rats. The effect of dietary MCT on adipocyte number may depend on the age of the animals.

Animal studies have already shown that insulin action is modulated by the type of fat (34). In these studies, saturated fat significantly increased insulin resistance, and unsaturated FA significantly improved it (35). MCT used in this study contained medium chain length saturated FA. However, this study

showed improved glucose tolerance in rats fed a diet containing MCT, compared with LCT rich in unsaturated FA. Previous studies also demonstrated that dietary MCT improve glucose tolerance in rats and humans (5,6). These results indicate the possibility of modulating insulin sensitivity by changing the length of the dietary saturated FA.

Little is known about the mechanism of higher adiponectin expression by suppression of adipocyte hypertrophy. In this study, mRNA levels related to adiponectin gene expression were determined. RXR and PPARγ mRNA levels were significantly higher in the rats fed the MCT diet. The PPARγ/RXR heterodimer directly bound to the peroxisome proliferator-activated receptor-responsive element (PPRE) and increased the promoter activity in cells. Previous studies demonstrated that TNF-α suppresses the gene expression of PPARγ and adiponectin (27,36). The TNF-α mRNA level in perirenal adipose tissue was not affected by the type of dietary fat in the present study. A future study should be designed to clarify the mechanism.

It has been reported that serum leptin concentration is positively correlated with subcutaneous fat (37). It must be noted that serum leptin levels did not seem affected despite the lower body fat in rats fed the MCT diet. However, the reason for this contradiction is unknown.

In conclusion, we demonstrated higher serum adiponectin levels and higher levels of adiponectin mRNA in perirenal adipose tissue in rats fed a diet containing MCT as compared with LCT. These findings suggest that dietary MCT, compared with LCT, causes higher serum adiponectin level with transcriptional activation of the adiponectin gene in rats. We speculate that improved glucose tolerance in rats fed an MCT diet may be, at least in part, ascribed to higher serum adiponectin level. In this study, we investigated the effect of a diet containing 20% of MCT on adiponectin in rats. It is difficult to administer a 20% MCT diet to human. Further study on the effects of partial substitution of MCT for LCT is necessary.

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Vitamin A Deficiency Enhances Docosahexaenoic and Osbond Acids in Liver of Rats Fed an α-Linolenic Acid-Adequate Diet

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ABSTRACT: The synthesis of docosahexaenoic (DHA, 22:6n-3) and Osbond acid (OA, 22:5n-6) is regulated by the heterodimer of peroxisome proliferator-activated receptor and retinoid X receptor (RXR). 9-*Cis* retinoic acid, a metabolite of vitamin A, is the most potent ligand of RXR. We tested whether vitamin A deficiency impairs DHA and OA synthesis in rats fed a vitamin A- and α-linolenic acid (ALA)-sufficient (VASALAS), vitamin A-sufficient and ALA-deficient (VASALAD), vitamin A–deficient and ALA-sufficient (VADALAS), or vitamin A- and ALA-deficient (VADALAD) diet. After 7 wk of feeding, liver and colon choline (CPG) and ethanolamine (EPG) phosphoglyceride FA were analyzed. The VADALAS compared with the VASALAS rats had elevated levels of both DHA ($P < 0.05$) and OA ($P < 0.005$) in liver CPG and EPG. In contrast, the VADALAD group had a lower DHA ($P < 0.01$) and higher OA ($P < 0.005$) level in CPG and EPG of both tissues than their VASALAD counterparts. ALA deficiency reduced DHA and enhanced OA levels in liver and colon CPG and EPG in both the vitamin A-sufficient (VASALAS vs. VASALAD) and -deficient (VADALAS vs. VADALAD) rats ($P < 0.005$). The study demonstrates that ALA deficiency reduced DHA and enhanced OA levels in tissue membranes, and dietary vitamin A deficiency has a profound effect on membrane DHA and OA in rat tissues. Both vitamin A and DHA are involved in a myriad of vital physiological functions pertaining to growth and development and health. Hence, there is a need for a further study to unravel the mechanism by which vitamin A influences membrane DHA and OA.

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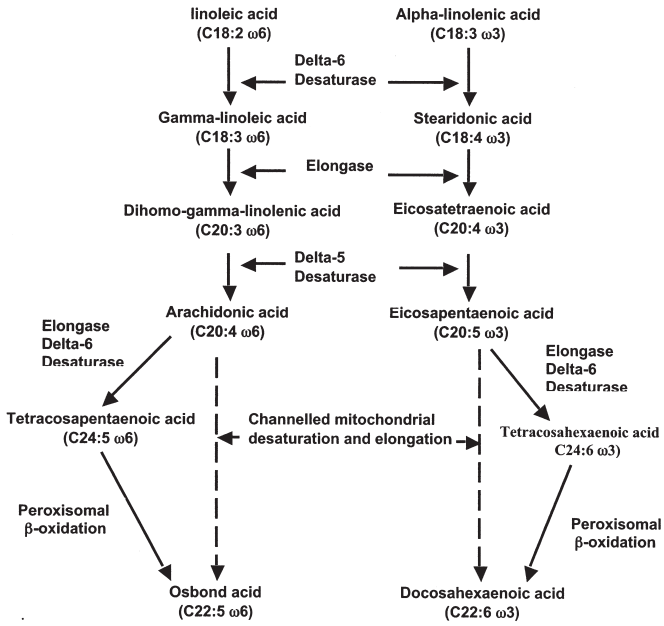
DHA (20:6n-3) is a major constituent of neural and retinal membrane lipids (1,2). In addition, it is present in appreciable proportions in other cell and subcellular membranes (3,4).

Membrane DHA is obtained either preformed from animal products, particularly from fish, shellfish, and fish oil, or from α-linolenic acid (ALA, 18:3n-3) by *de novo* synthesis. It is generally accepted that the synthesis of DHA from ALA involves a series of elongation and desaturation steps in endoplasmic reticulum followed by a final single step β-oxidation reaction in the peroxisomes (5–7) (Scheme 1). Osbond acid (OA, 22:5n-6) is also thought to be synthesized from its precursor FA, linoleic acid (LA, 18:2n-6), by the same pathway, following elongation, desaturation, and β-oxidation reactions.

The activation of peroxisome proliferator-activated (PPAR) and retinoid X (RXR) receptors is imperative for the proliferation of peroxisomes and activation of the key enzymes involved in peroxisomal β-oxidation. On activation by a specific ligand, PPAR form a heterodimer complex (PPAR-RXR) with activated RXR and subsequently bind to specific DNA sequences and initiate inducible transcriptional activity (8–11). The heterodimer PPAR-RXR also has been shown to up-regulate the activity of Δ6 and Δ5 desaturases (12), enzymes that are vital for the synthesis of the long-chain n-6 and n-3 FA, including DHA and OA.

PPAR are members of the intracellular type II nuclear hormone receptor superfamily. Three subtypes have been identified, $PPAR_{\alpha}$, $PPAR_{\beta}/PPAR_{\delta}$, and $PPAR_{\gamma}$ (13). They are acti-



SCHEME 1

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Abbreviations: ALA, α-linolenic acid; CPG, choline phosphoglyceride; EPG, ethanolamine phosphoglyceride; OA, Osbond acid (= docosapentaenoic acid); PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; VADALAD, vitamin A- and ALA-deficient; VADALAS, vitamin A-deficient and ALA-sufficient; VASALAD, vitamin A-sufficient and ALA-deficient; VASALAS, vitamin A- and ALA-sufficient.

TABLE 1
Gross Nutrient Composition of the Vitamin A-Sufficient (VAS) and -Deficient (VAD) Diets

Ingredients	VAD (g/kg diet)	VAS (g/kg diet)
Casein, vitamin-free	200.0	200.0
DL-Methionine	3.0	3.0
Sucrose	437.98	437.98
Corn starch	200.0	200.0
Oils	90.0	90.0
Cellulose (fiber)	30.0	30.0
Mineral mix, AIN-76 (170915)	35.0	35.0
Calcium carbonate, $CaCO_3$	4.0	4.0
Vitamin A (IU)	0	19825
Vitamin mix, Teklad (40060)	10.0	10.0
Ethoxyquin (antioxidant)	0.016	0.016

TABLE 2
Percent FA Composition of the α-Linolenic Acid (ALA)-Deficient (ALAD) and -Sufficient (ALAS) Diets

FA	ALAD (% of total FA)	ALAS (% of total FA)
14:0	1.12	0.976
16:0	21.85	19.44
18:0	2.31	2.49
18:1n-9	18.81	19.25
18:2n-6	53.09	49.21
18:3n-3	0.31	6.82
18:2n-6/18:3n-3	170:1	7:1

vated by polyunsaturated, conjugated, and branched FA, eicosanoids, and synthetic ligands such as fibrates (14). In contrast to PPAR, RXR is activated primarily by 9-*cis* retinoic acid, which is a metabolite of vitamin A (8,9), and by phytanic acid with a low affinity (15).

Previously (16), we showed that vitamin A deficiency enhances OA and reduces DHA levels in liver and colon choline (CPG) and ethanolamine (EPG) phosphoglycerides in rats fed a diet deficient in n-3 and sufficient in n-6 FA. The finding was rather intriguing since vitamin A deficiency would have been expected to reduce the levels of both DHA and OA owing to a reduction of activation of RXR and consequently impaired peroxisomal proliferation. In the current study, we have investigated the effect of vitamin A deficiency on levels of DHA and OA in liver and colon phosphoglycerides in rats fed a diet sufficient or deficient in n-3 FA.

EXPERIMENTAL PROCEDURES

Animals. Pathogen-free, weanling male Wistar rats (n = 24) were obtained from the Harlan laboratory, The Weizmann Institute of Science, Rehovot, Israel. They were housed in metal cages in a room with controlled temperature (22 ± 2°C), RH (65 ± 5%), and light (0800–2000 h).

Diets. Twenty-four rats were randomly distributed into four groups and fed one of the following diets: vitamin A- and α-linolenic acid (ALA)-deficient (VADALAD, n = 6), vitamin A-deficient and ALA-sufficient (VADALAS, n = 6), vitamin A-sufficient and ALA-deficient (VASALAD, n = 6), or vitamin A- and ALA-sufficient (VASALAS, n = 6). The diet, which was obtained from ICN Nutritional Biochemicals (Costa Mesa, CA) (cat. no. 960220) contained 9% fat, 20% protein, and 60% carbohydrate (Tables 1 and 2). With the exceptions of vitamin A and n-6 and n-3 FA, the four diets had optimal and comparable amounts of vitamins and trace elements. The linoleic acid/ALA ratio of the ALA-sufficient diet was 7:1 and that of the ALA-deficient, 170:1. The rats had free access to their respective diets and water. Their food consumption was monitored daily, and they were weighed every other day.

After they were fed for 7 wk, at which time the liver vitamin A concentration of the deficient group was lower than 5 μg/g liver, the rats were sacrificed by decapitation and colon and liver tissues were removed for analyses. All procedures were conducted with full compliance to the guidelines of the Ethics Committee and Policy of Animal Care and Use of the Hebrew University.

Analysis of lipids and FA. A method modified from Folch *et al.* (17) was used to extract liver and colon total lipids. The tissues were homogenized in chloroform and methanol (2:1 vol/vol) containing 0.01% BHT as an antioxidant under N_2. Phosphoglyceride classes were separated by TLC on silica gel plates (Merck KGaA, Darmstadt, Germany) by the use of these developing solvents: Chloroform, methanol, and water (60:30:4 by vol) containing 0.01% BHT. CPG and EPG bands were detected by spraying with a methanolic solution of 2,7-dichlorofluorescein (0.01% wt/vol) and identified by the use of authentic standards.

FAME were prepared by heating the CPG and EPG with 5 mL of 15% acetyl chloride in methanol in a sealed vial at 70°C for 3 h under N_2. FAME were separated by a gas–liquid chromatograph (HRGC MEGA-2 Series; Fisons Instruments, Milan,Italy) fitted with a BP-20 capillary column (30 m × 0.32 mm i.d., 0.25 μm film; SGE Ltd., Milton Keynes, United Kingdom). Hydrogen was used as a carrier gas. It has a broad minimum van Deemter profile, which is vital for optimal performance, as compared with helium and nitrogen. In addition, it has faster diffusion and lower viscosity than the latter gases. The injector, oven, and detector temperatures were 235, 210, and 260°C. The FAME were identified by comparison of retention times with authentic standards and interpretation of ECL values. Peak areas were quantified by computer software (EZChrom Chromatography Data System; Scientific Software, Inc., San Ramon, CA).

Analysis of liver vitamin A. The liver specimen, 100 mg, was thoroughly minced, flushed with nitrogen, and saponified with a 50% ethanolic potassium hydroxide (BDH Chemicals, Poole, United Kingdom) for 25 min at 60°C. The saponified sample was cooled, diluted with de-ionized water, and neutralized with hydrochloric acid (BDH Chemicals). Extraction of vitamin A was carried out with 2 mL of hexane for 5 min on a Rotamixer Shaker (Baird and Tatlock, Ramford, Essex, United Kingdom). After centrifugation at 800 × g for 15 min, the upper organic layer containing vitamin A was transferred to a brown glass tube and subsequently evaporated to dryness on a water bath at 37°C under a stream of nitrogen. The re-

TABLE 3
Food Consumption, Weight Gain, Liver Vitamin A Concentration, and Somatic Index[a]

	VADALAS (n = 6)	VASALAS (n = 6)	VADALAD (n = 6)	VASALAD (n = 6)
Food consumption (g/d)	13.6 ± 1.1	17.7 ± 1.1	13.5 ± 0.9	18.6 ± 1.4
Weight gain (g/wk)	32.9 ± 5.2	41.2 ± 5.2	32.1 ± 4.4	41.5 ± 7.8
Liver vitamin A (μg/g liver)	<5	271.1	<5	296.4
Liver somatic index (liver/body weight)	3.4 ± 0.4	3.3 ± 0.3	3.4 ± 0.3	3.7 ± 0.6

[a]VADALAD, vitamin A- and ALA-deficient; VADALAS, vitamin A-deficient and ALA-sufficient; VASALAD, vitamin A-sufficient and ALA-deficient; VASALAS, vitamin A- and ALA-sufficient.

sulting vitamin A residue was redissolved in 100 μL methanol (BDH Chemicals) and a 25-μL aliquot taken for analysis. The entire extraction procedure was carried out under subdued light.

The extracted vitamin A was separated by HPLC (Agilent 1100 series; Agilent Technologies, Waldbronn, Germany) connected to a 250 × 4.6 mm and a 5 μ SphereClone ODS reversed-phase column (Phenomenex, Macclesfield, Cheshire, United Kingdom) and detected at 325 nm with a multiple wavelength diode array detector (Agilent 1100 series). It was eluted with acetonitrile/dichloromethane/methanol (75:15:10). Retinyl acetate was used as an internal standard. Vitamin A concentration was computed from a linear graph of retinol external standards. ChemStation data system version A10.01 (Agilent Technologies) was used for peak area measurement and quantification.

Data analyses. The data are expressed as mean ± SD. The Kruskal–Wallis one-way ANOVA nonparametric method was used to compare liver and colon FA levels of the four dietary groups. Differences between two dietary groups were analyzed by the Mann–Whitney U nonparametric test. All the statistical analyses were performed by the use of SPSS software for Windows, Release 10 (SPSS, Chicago, IL).

RESULTS

Food consumption and weight gain. The food consumption of the VADALAD and VADALAS groups was lower ($P < 0.05$) than that of the VASALAD and VASALAS groups (Table 3). No difference in food consumption was found between VADALAD and VADALAS, and between VASALAS and VASALAD, respectively.

Consistent with change of the food consumption, the weight gain of the VADALAD and VADALAS groups was lower ($P < 0.005$) than that of the VASALAD and VASALAS groups (Table 3). No difference in weight gain was observed between VADALAD and VADALAS, and between VASALAS and VASALAD, respectively. Moreover, no difference in liver somatic index was found among VADALAS, VASALAS, VADALAD, and VASALAD groups.

TABLE 4
Liver Choline Phosphoglyceride FA (%) of the Rats Fed an ALA-Adequate or -Deficient Diet With or Without Vitamin A[a]

FA	VADALAS (n = 6)	VASALAS (n = 6)	VADALAD (n = 6)	VASALAD (n = 6)
16:0	26.68 ± 1.57^{b,c}	20.42 ± 1.33^{a,c,d}	24.67 ± 0.86^{a,b}	25.19 ± 1.98^{b}
18:0	16.82 ± 2.46^{d}	15.35 ± 2.38^{d}	16.32 ± 1.37^{d}	20.85 ± 1.57^{a,b,c}
Σ Saturates	43.77 ± 1.74^{b,c,d}	36.15 ± 1.10^{a,c,d}	41.30 ± 1.14^{a,b,d}	46.31 ± 1.10^{a,b,c}
18:1n-9	5.00 ± 1.06^{b,d}	6.61 ± 0.89^{a,d}	5.89 ± 0.34^{d}	3.60 ± 0.70^{a,b,c}
18:1n-9 +18:1n-7	7.44 ± 1.75^{b}	11.31 ± 1.78^{a,c,d}	8.56 ± 1.87^{b,d}	5.80 ± 0.74^{b,c}
Σ Monoenes	8.74 ± 1.27^{b,d}	13.08 ± 2.46^{a,c,d}	9.44 ± 2.10^{b,d}	6.68 ± 0.93^{a,b,c}
18:2n-6	16.01 ± 2.80^{b}	21.43 ± 2.09^{a,c,d}	15.86 ± 2.60^{b}	14.56 ± 1.37^{b}
18:3n-6	0.15 ± 0.02^{b,c}	0.35 ± 0.06^{a,c,d}	0.42 ± 0.04^{a,b,d}	0.19 ± 0.05^{b,c}
20:3n-6	0.46 ± 0.08^{d}	0.56 ± 0.16^{c,d}	0.37 ± 0.06^{b,d}	0.19 ± 0.05^{a,b,c}
20:4n-6	19.42 ± 2.14^{d}	21.76 ± 1.78^{d}	21.45 ± 1.47^{d}	26.27 ± 1.68^{a,b,c}
22:4n-6	0.24 ± 0.04^{b,c}	0.13 ± 0.03^{a,c,d}	0.97 ± 0.20^{a,b,d}	0.25 ± 0.03^{b,c}
22:5n-6	0.55 ± 0.07^{b,c,d}	0.06 ± 0.05^{a,c,d}	7.01 ± 1.10^{a,b,d}	1.80 ± 0.41^{a,b,c}
Σ n-6	37.40 ± 1.32^{b,c,d}	44.95 ± 2.03^{a}	46.65 ± 1.79^{a,d}	43.52 ± 1.24^{a,c}
18:3n-3	0.35 ± 0.19^{b}	0.81 ± 0.27^{a}	—^{b}	—
20:5n-3	0.05 ± 0.03^{b}	0.47 ± 0.10^{a}	—	—
22:5n-3	0.72 ± 0.15^{b,c,d}	0.48 ± 0.09^{a,c,d}	0.10 ± 0.03^{a,b}	0.12 ± 0.01^{a,b}
22:6n-3	6.01 ± 0.88^{b,c,d}	2.69 ± 0.75^{a,c,d}	0.68 ± 0.09^{a,b,d}	1.65 ± 0.25^{a,b,c}
Σ n-3	7.05 ± 0.69^{b,c,d}	4.45 ± 0.56^{a,c,d}	0.80 ± 0.09^{a,b,d}	1.83 ± 0.24^{a,b,c}

[a]A superscript letter denotes a significant difference from the corresponding group at $P < 0.05$ level. For abbreviations see Tables 2 and 3 .
[b]—, trace.

TABLE 5
Liver Ethanolamine Phosphoglyceride FA (%) of Rats Fed Adequate or Deficient ALA Diets With or Without Vitamin A[a]

FA	VADALAS ($n = 6$)	VASALAS ($n = 6$)	VADALAD ($n = 6$)	VASALAD ($n = 6$)
16:0	22.32 ± 0.69[b,c,d]	16.83 ± 0.86[a]	18.61 ± 1.72[a,d]	16.75 ± 1.08[a,c]
18:0	24.45 ± 1.07[b,c,d]	21.90 ± 1.49[a,d]	22.60 ± 2.18[a,d]	28.85 ± 1.98[a,b,c]
Σ Saturates	47.09 ± 0.83[b,c]	38.83 ± 1.95[a,c,d]	41.27 ± 1.20[a,b,d]	45.72 ± 2.37[b,c]
18:1n-9	2.88 ± 0.46[d]	3.27 ± 0.33[d]	3.03 ± 0.21[d]	1.92 ± 0.17[a,b,c]
18:1n-9 + 18:1n-7	4.17 ± 0.59[b,d]	6.59 ± 1.16[a,c,d]	4.33 ± 0.47[b,d]	3.13 ± 0.35[a,b,c]
Σ Monoenes	4.44 ± 0.58[b]	7.09 ± 0.97[a,c,d]	4.65 ± 0.58[b,d]	3.35 ± 0.42[b,c]
18:2n-6	5.38 ± 0.98[b,d]	7.88 ± 1.22[a,c]	5.73 ± 1.69[b,d]	7.79 ± 1.16[a,c]
18:3n-6	0.05 ± 0.01[b,c,d]	0.12 ± 0.05[a,c]	0.28 ± 0.02[a,b,d]	0.10 ± 0.03[a,c]
20:3n-6	0.25 ± 0.03[b]	0.46 ± 0.14[a,c,d]	0.29 ± 0.08[b]	0.25 ± 0.08[b]
20:4n-6	20.44 ± 0.78[b,d]	27.14 ± 2.02[a,c]	21.77 ± 1.55[b,d]	28.51 ± 1.46[a,c]
22:4n-6	0.78 ± 0.11[b,c]	0.55 ± 0.05[a,c,d]	2.56 ± 0.44[a,b,d]	0.97 ± 0.14[b,c]
22:5n-6	1.20 ± 0.14[b,c,d]	0.27 ± 0.06[a,c,d]	16.84 ± 1.52[a,b,d]	4.40 ± 0.61[a,b,c]
Σ n-6	28.26 ± 0.26[b,c,d]	36.83 ± 2.41[a,c,d]	47.81 ± 0.9[a,b,d]	42.23 ± 2.13[a,b,c]
18:3n-3	0.10 ± 0.04	0.13 ± 0.07	—[b]	—
20:5n-3	0.08 ± 0.04[b]	0.55 ± 0.21[a]	—	—
22:5n-3	1.42 ± 0.31[c,d]	1.40 ± 0.30[b,c,d]	0.20 ± 0.06[a,b,d]	0.43 ± 0.13[a,b,c]
22:6n-3	14.08 ± 1.20[b,c,d]	10.98 ± 1.93[a,c,d]	1.74 ± 0.20[a,b,d]	4.44 ± 0.49[a,b,c]
Σ n-3	15.50 ± 1.13[b,c,d]	13.05 ± 1.63[a,c,d]	1.94 ± 0.20[a,b,d]	4.88 ± 0.48[a,b,c]

[a,b]For superscripts and abbreviations see Tables 3 and 4.

Liver FA. (i) CPG. The FA composition of liver CPG is presented in Table 4. The mean DHA level in CPG of the VADALAS group was significantly higher than that of the VASALAS ($P < 0.05$), VASALAD ($P < 0.005$), and VADALAD ($P < 0.005$) groups. In addition, the VADALAD rats had a lower level of DHA in CPG compared with the VASALAD ($P < 0.05$) and VASALAS ($P < 0.005$) groups. Of the two vitamin A-sufficient groups, VASALAS and VASALD, the former had a higher DHA level ($P < 0.01$).

As was expected, the proportion of OA, which is a biochemical marker of n-3 FA deficiencies, was increased by ALA deficiency both in the vitamin A-sufficient (VASALAD vs. VASALAS, $P < 0.005$) and -deficient (VADALAD vs. VADALAS, $P < 0.005$) rats. Surprisingly, vitamin A deficiency enhanced the level of OA in the ALA-sufficient (VADALAS vs. VASALAS; $P < 0.005$) and ALA-deficient (VADALAD vs. VASALAD; $P < 0.005$) groups.

(ii) EPG. The mean liver EPG FA composition is given in Table 5. Consistent with the manifestations of n-3 FA insufficiencies, ALA deficiency decreased DHA level in both the vitamin A-sufficient (VASALAD vs. VASALAS, $P < 0.005$) and -deficient (VADALAD vs. VADALAS, $P < 0.005$) groups. The decrease in DHA was associated with a concomitant increase in OA (VASALAD vs. VASALAS, $P < 0.005$) and (VADALAD *vs* VADALAS, $P < 0.005$). As in CPG, the EPG of vitamin A-deficient rats regardless of their ALA status had a significantly elevated proportion of OA.

Colon FA. (i) CPG. Table 6 shows colon CPG FA of the four groups of rats. The VADALAS animals had a higher level of DHA than those of the VASALAS ($P < 0.05$), VADALAD ($P < 0.01$), and VASALAD ($P < 0.005$) rats. In contrast to the VADALAS, the VADALAD rats had reduced DHA compared with the VASALAD rats ($P < 0.01$). Vitamin A deficiency increased the level of OA in the ALA-deficient, VADALAD, ($P < 0.05$) but not in the ALA sufficient, VADALAS, rats.

(ii) EPG. Percent FA composition of colon EPG is given in Table 7. ALA deficiency significantly reduced the level of DHA in the vitamin A-sufficient (VASALAD vs. VASALAS, $P < 0.005$) and -deficient (VADALAD vs. VADALAS, $P < 0.005$) rats. Vitamin A deficiency reduced DHA in the ALA-deficient (VADALAD vs. VASALAD) but not in the ALA-sufficient (VADALAS vs. VASALAS, $P > 0.05$) rats. The level of OA significantly increased in the ALA-deficient compared with the ALA-sufficient groups (VADALAD vs. VADALAS, $P < 0.005$; VASALAD vs. VASALAS, $P < 0.005$). Although vitamin A deficiency in both the VADALAS and VADALAD groups was associated with an increase in OA, the difference with their corresponding vitamin A-sufficient counterparts did not reach a stastically significant level (VADALAS vs. VASALAS, VADALAD vs. VASALAD, $P = 0.078$).

DISCUSSION

Consistent with our previous findings in rats (18) and chicks (19), the vitamin A-deficient groups, regardless of the ALA content of their diet, consumed less food compared with their vitamin A-sufficient counterparts. The reduction of food intake and consequent lower weight gain of the vitamin A-deficient animals were most likely due to vitamin A deficiency-induced atrophy of the gustatory apparatus (20,21) and a loss of taste (20–22). The increase in OA and the concomitant reduction of DHA in the ALA-deficient rats, regardless of vitamin A status, suggest that dietary ALA and LA may be the primary sources of membrane DHA and OA, respectively. These findings are analogous to some reports about n-3 FA deficiencies (23,24).

This partial investigation of the effect of vitamin A defi-

TABLE 6
Colon Choline Phosphoglyceride FA (%) of Rats Fed ALA-Adequate or -Deficient Diet With or Without Vitamin A[a]

FA	VADALAS (n = 6)	VASALAS (n = 6)	VADALAD (n = 6)	VASALAD (n = 6)
16:0	31.17 ± 3.20	32.75 ± 2.71	32.31 ± 3.03	28.37 ± 3.50
18:0	10.97 ± 1.83	10.91 ± 0.99	11.51 ± 1.38	11.55 ± 1.76[c]
Σ Saturates	43.37 ± 2.37	44.86 ± 2.20[d]	44.68 ± 2.45[d]	40.43 ± 1.96[b,c]
18:1n-9	10.27 ± 1.47	10.41 ± 1.84	10.16 ± 1.44[d]	12.05 ± 0.53[c]
18:1n-9 + 18:1n-7	15.03 ± 2.06	15.26 ± 2.22	14.68 ± 1.59[d]	17.08 ± 0.68[c]
Σ Monoenes	17.23 ± 2.05	17.82 ± 2.53	16.43 ± 1.67[d]	18.94 ± 0.92[c]
18:2n-6	13.29 ± 2.05	14.02 ± 1.46	12.14 ± 2.20[d]	15.47 ± 2.23[c]
18:3n-6	0.18 ± 0.04[d]	0.14 ± 0.04	0.17 ± 0.04[d]	0.11 ± 0.03[a,c]
20:3n-6	2.19 ± 0.22[b,d]	1.66 ± 0.33[a]	1.94 ± 0.40	1.14 ± 0.16[a]
20:4n-6	12.38 ± 0.74[c,d]	11.43 ± 1.11[c,d]	14.17 ± 0.92[a,b]	15.16 ± 2.13[a,b]
22:4n-6	1.01 ± 0.13[d]	0.99 ± 0.06[d]	1.28 ± 0.26	1.44 ± 0.16[a,b]
22:5n-6	0.16 ± 0.04[c,d]	0.11 ± 0.04[c,d]	0.74 ± 0.16[a,b,d]	0.48 ± 0.04[a,b,c]
Σ n-6	30.18 ± 1.73[d]	29.29 ± 1.46[d]	31.39 ± 1.86[d]	35.32 ± 2.63[a,b,c]
18:3n-3	0.29 ± 0.08[b]	0.54 ± 0.11[a]	—[b]	—
20:5n-3	0.19 ± 0.05	0.25 ± 0.06	—	—
22:5n-3	0.29 ± 0.02[c,d]	0.33 ± 0.07[c,d]	0.05 ± 0.02[a,b]	0.11 ± 0.07[a,b]
22:6n-3	1.09 ± 0.26[b,c,d]	0.70 ± 0.16[a,c,d]	0.35 ± 0.06[a,b,d]	0.46 ± 0.06[a,b,c]
Σ n-3	1.86 ± 0.22[c,d]	1.82 ± 0.23[c,d]	0.44 ± 0.07[a,b,d]	0.67 ± 0.18[a,b,c]

[a,b]For superscripts and abbreviations see Tables 3 and 4.

ciency on FA composition in liver membrane lipids of rats fed an ALA-deficient diet, consistent with our previous study (16), demonstrated that vitamin A deficiency leads to a reduction in DHA and a concomitant increase in OA in liver CPG and EPG. The reduction in DHA was predicted since vitamin A deficiency was expected to impair peroxisomal proliferation and inhibit the activity of acyl-CoA oxidase owing to an insufficiency of 9-*cis* retinoic acid, which is the potent ligand of RXR. However, the vitamin A deficiency-induced increase in OA was unexpected and rather intriguing. Both DHA and OA are thought to be synthesized by a common shared microsomal-peroxisomal pathway involving chain elongation and desaturation reactions in the endoplasmic reticulum followed by a final one-step β-oxidation reaction in the peroxisomes (5,25). Consequently, an impairment of peroxisomal proliferation would have been expected to lead to a reduction in both OA and DHA. The contrasting effects of vitamin A and ALA deficiency on the proportions of DHA and OA in liver CPG and EPG seemed to lend credence to the proposition that the two FA are synthesized by independent pathways involving n-6- and n-3-specific enzymes (6,7) or to the suggestion that the desaturation of n-6 and n-3 involves two distinct Δ6 desaturases (26). Indeed, Infante *et al.* (27) reported that the straight-chain acyl-CoA oxidase knockout mouse had decreased DHA and increased threefold OA in liver. Our enhancement of OA and reduction of DHA levels in VADALAD rats, similar to their findings, can be explained by hypothesizing that DHA and OA are synthesized by a carnitine-dependent multifunctional mitochondrial synthase residing in the outer mitochondrial membrane, and beyond this, that there is another redundant microsomal pathway that is only suitable for OA synthesis (6,7). In contrast to the rats fed the VADALAD diet, which had reduced DHA and enhanced OA levels, the levels of both DHA and OA were enhanced in the rats that were fed VADALAS diet. Paradoxically, this finding is consistent with the conventional microsomal-peroxisomal and additional carnitine-dependent multifunctional mitochondrial pathways (5–7,26). These effects of vitamin A deficiency on membrane DHA and OA may have been mediated by enhanced peroxisomal proliferation and the consequential activation of peroxisomal enzymes, including acyl-CoA oxidase, activity of Δ6 and Δ5 desaturases, or incorporation. The current wisdom is that peroxisomal proliferation is mediated by PPAR-RXR complex after the activation of RXR by 9-*cis* retinoic acid, and it is difficult to envisage how this could be induced under vitamin A deficiency. However, the studies that reported that 9-*cis* retinoic acid is a potent ligand of RXR were conducted *in vitro* (28–30). Consequently, it is possible that the finding may not be portable to an *in vivo* physiological environment. Indeed, Lawrence *et al.* (31) have reported significantly higher induction of peroxisomal β-oxidation in vitamin A-deficient than -sufficient rats that were treated by the potent peroxisomal proliferator, nafenopin. Similarly, in a communication by Sohlenus *et al.* (32), the vitamin A-deficient mice untreated by the peroxisomal proliferator had about 40% higher volume and number of liver peroxisomes compared with their sufficient counterparts; the authors did not discuss this finding. Interestingly, Werner and DeLuca (33) were unable to detect 9-*cis* retinoic acid in tissues of vitamin A-deficient rats that were given a physiological amount of radioactive all-*trans* retinol. It is plausible that vitamin A deficiency may enhance the synthesis of OA and/or DHA through the induction of peroxisomal proliferation and the activation of β-oxidation without the involvement of 9-*cis* retinoic acid. On the other hand, both DHA and OA can be synthesized in another carnitine-dependent multifunctional mitochondrial pathway (6,7,27). There is evidence that DHA was still the predom-

TABLE 7
Colon Ethanolamine Phosphoglyceride FA (%) of Rats Fed ALA-Adequate or -Deficient Diet With or Without Vitamin A[a]

FA	VADALAS (n = 6)	VASALAS (n = 6)	VADALAD (n = 6)	VASALAD (n = 6)
16:0	5.45 ± 0.63	6.02 ± 0.57[d]	5.52 ± 0.50[d]	4.81 ± 0.41[b,c]
18:0	11.64 ± 1.07	11.61 ± 0.56	11.45 ± 1.10	11.31 ± 0.68
Σ Saturates	17.35 ± 1.40	17.91 ± 0.31[c,d]	16.98 ± 0.93[b]	16.25 ± 0.79[b]
18:1n-9	5.96 ± 0.62	6.40 ± 0.71	5.71 ± 0.30	5.78 ± 0.55
18:1n-9 + 18:1n-7	7.30 ± 0.69	7.99 ± 0.80	7.24 ± 0.37	7.19 ± 0.61
Σ Monoenes	8.10 ± 0.86	8.95 ± 0.97[c]	7.91 ± 0.58[b]	8.06 ± 0.71
18:2n-6	4.91 ± 0.87	5.36 ± 0.86	6.32 ± 1.61[d]	4.72 ± 0.77[c]
18:3n-6	0.07 ± 0.02[c,d]	0.06 ± 0.02	0.04 ± 0.01[a]	0.05 ± 0.01[a]
20:3n-6	2.01 ± 0.26[d]	1.79 ± 0.43	2.12 ± 0.33[d]	1.38 ± 0.41[a,c]
20:4n-6	30.44 ± 1.67	28.17 ± 1.70[c,d]	32.02 ± 1.18[b]	33.08 ± 2.64[b]
22:4n-6	4.27 ± 0.48[b,c,d]	4.82 ± 0.29[a,d]	5.41 ± 0.52[a,d]	6.63 ± 0.54[a,b,c]
22:5n-6	0.74 ± 0.13[c,d]	0.58 ± 0.08[c,d]	3.44 ± 0.64[a,b]	2.86 ± 0.54[a,b]
Σ n-6	42.68 ± 2.21[c,d]	41.23 ± 1.48[c,d]	49.09 ± 1.74[a,b]	49.06 ± 2.44[a,b]
18:3n-3	0.12 ± 0.04[b]	0.36 ± 0.11[a]	—[b]	—
20:5n-3	0.55 ± 0.12	0.58 ± 0.07	—	—
22:5n-3	0.96 ± 0.12[b,c,d]	1.32 ± 0.18[a,c,d]	0.08 ± 0.01[a,b,d]	0.32 ± 0.06[a,b,c]
22:6n-3	3.30 ± 0.64[c,d]	3.26 ± 0.41[c,d]	0.69 ± 0.10[a,b,d]	1.72 ± 0.31[a,b,c]
Σ n-3	4.91 ± 0.64[c,d]	5.52 ± 0.41[c,d]	0.79 ± 0.11[a,b,d]	2.09 ± 0.36[a,b,c]

[a,b]For superscripts and abbreviations see Tables 3 and 4.

inant FA in straight-chain acyl-CoA oxidase null mice, and its amount was comparable to that in their corresponding wild-type animals (27). The characteristics of these multifunctional pathways are that the enzyme-bound products are recycled back as substrates until the desired final product is formed. In these recycling reactions, alternative *cis*-desaturations and elongation of the enzyme-bound acyl intermediates would introduce the methylene-interrupted double-bond structure of the final products. The enhanced DHA and OA suggest that a termination reaction catalyzed by an acyl carrier protein-connected carnitine acyltransferase might be preferential for the release of DHA or OA from enzyme in vitamin A-deficient rats.

Although vitamin A deficiency has been shown to enhance the activity of Δ5 (34,35) and Δ9 (36) desaturases, such influence was not apparent in this study. This difference could be a reflection of the rat strain (37) or diet used.

From the present study, it is evident that dietary vitamin A deficiency has a profound effect on membrane DHA and OA in rat tissues. It increases the proportions of OA consistently regardless of the level of dietary ALA. In contrast, the effect on DHA is dependent on dietary ALA level; it is reduced when dietary ALA is limiting and is enhanced when ALA is adequate. Both vitamin A and DHA are involved in a myriad of vital physiological functions pertaining to growth and development and health. Hence there is a need for a further study to unravel the mechanism by which vitamin A influences membrane DHA and its n-6 counterpart, OA.

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Effects of Geographic Source, Rearing System, and Season on the Nutritional Quality of Wild and Farmed *Perca fluviatilis*

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ABSTRACT: The effects of season, geographic source (Lake Geneva, Rhine River), and rearing system (extensive, semiextensive, and intensive systems) on the lipid content and FA composition of fillets of *Perca fluviatilis* were studied. Significant differences in the total lipid content were found between fish coming from the Rhine River and Lake Geneva (1.21 and 1.48%, respectively). Seasonal effects were investigated quarterly for perch sampled in the Rhine River. Intensively reared perch displayed a higher lipid content (1.48%) than the other farmed perch, i.e., 1.26% for a semiextensive system and 1.16% for an extensive system. No significant difference in lipid content was found (i) between lacustrine fish and intensively reared fish or (ii) among fish from the Rhine River and the semiextensive or extensive rearing systems. The main FA were 22:6n-3 (DHA, 21.3–37.1% of total FA), 16:0 (17.7–20.2%), 20:5n-3 (EPA, 9.2–13.2%), 18:1 (8.0–11.5%), 20:4n-6 [arachidonic acid (ARA), 1.9–10.7%], 16:1 (4.3–6.0%), and 18:2n-6 (2.1–6.0%). In comparison with perch coming from the Rhine River, the lacustrine fish were characterized by higher total n-6 PUFA and a lower proportion of both total monounsaturated FA (MUFA) and total n-3 PUFA. Among rearing systems, extensively farmed fish had higher n-6 PUFA and lower n-3 PUFA contents. Wild fish showed higher ARA and 18:2n-6 than farmed fish. They also had significantly more EPA (12.5–13.2%) than farmed perch (9.2–10.9%). For DHA, no difference existed between (i) the lacustrine fish (31.9% of total FA) and the intensively reared fish (33.0%) and (ii) the Rhine (37.1%) and semiextensively reared fish (36%). Effects of size, diet composition, and environmental conditions on the total lipid contents and FA composition are discussed.

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Beneficial effects of fish consumption on human health, e.g., stroke reduction and prevention of hypertension, cancer, heart disease, or depression, have been demonstrated previously (1). These health benefits appear to be related to the high content of PUFA (15–36% of the total FA), particularly n-3 PUFA such as DHA (22:6n-3) and EPA (20:5n-3) (2). Clearly, both the FA composition and lipid content of fillets could be influenced by many parameters, such as genetic factors, geographic origin or domestication level, food availability and quality, and season (3–5). Moreover, it is well established that nutritional quality differs between wild and reared fish (2), but also among different rearing systems (6).

Aquaculture of the Eurasian perch, *Perca fluviatilis*, is a diversification route for inland aquaculture. Perch fillets are considered a dietetic product because of their low fat and high DHA contents (7). In addition, the Eurasian perch is very adaptable, which allows for a wide variety of rearing systems in aquaculture. However, the effects of such different rearing systems on the nutritional quality of perch still remain unclear. Moreover, perch is a very common species inhabiting very different biotopes and large geographic areas (8). All these different environmental conditions could be responsible for differences in the quality of perch at large. However, no information about the variability of the nutritional quality regarding the geographic source is currently available. The purpose of the present paper was threefold. First, it was intended to study the variability of fillet lipids and the FA contents of Eurasian perch from two geographic sources, namely, the Rhine River and Lake Geneva, and from three rearing systems, ranked as intensive, semiextensive, and extensive. Second, a comparison between wild and farmed perch was carried out. Third, the effect of season on the nutritional quality of wild perch was assessed. In addition, the relationship between the nutritional quality of fish and the composition of the fish diet was assessed using stable isotope analysis.

MATERIALS AND METHODS

Fish collection. Live fish from the Rhine River (Boven-Hardinxveld, The Netherlands) and Lake Geneva (Lugrin, France) were sampled in July and October of 2002 and in January and April of 2003. We assumed that differences in nutritional quality could be found in wild fish living in two very different natural systems. Thus, these two sites were selected on the basis of their very different physicochemical water parameters (9). Fish were collected according to the normal practice used by professional fishermen for perch fishing (net for Lake Geneva and fish pot for the Rhine River) to provide a wild reference base corresponding to the fish consumed in each area. Nets and fish pots were submerged for 3 to 5 d. Farmed perch were collected during the period of market consumption, i.e., in October 2003. They were sampled from three different rear-

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Abbreviations: ARA, arachidonic acid; MUFA, monounsaturated fatty acid(s); SFA, saturated fatty acid(s).

TABLE 1
Composition of the Artificial Food[a]

	Bio-Optimal ST	Ecolife 15
Protein (%)[b]	47.1 ± 0.4	44.6 ± 0.4
Lipid (%)[b]	10.0 ± 0.2	17.2 ± 0.2
Moisture (%)[b]	6.1 ± 0.1	5.1 ± 0.0
Ash (%)[b]	9.7 ± 0.1	7.1 ± 1.1
FA composition (% total FA)[c]		
14:0	5.0 ± 0.2	6.1 ± 0.1
16:0	14.4 ± 0.5	16.4 ± 0.0
18:0	2.7 ± 0.0	2.6 ± 0.0
16:1	5.7 ± 0.2	6.8 ± 0.0
18:1	11.7 ± 0.1	12.5 ± 0.1
20:1	5.6 ± 0.1	5.3 ± 0.1
22:1	7.3 ± 0.3	7.0 ± 0.1
18:2n-6	7.8 ± 0.1	6.0 ± 0.1
20:4n-6	1.0 ± 0.0	1.1 ± 0.0
18:3n-3	1.7 ± 0.0	1.6 ± 0.0
18:4n-3	1.7 ± 0.0	1.7 ± 0.0
20:5n-3	19.3 ± 0.2	18.6 ± 0.1
22:6n-3	13.1 ± 0.5	11.4 ± 0.2

[a]Each sample of food was analyzed in triplicate. Bio-Optimal ST and Ecolife 15 were from BioMar (Brande, Denmark).
[b]Results are expressed as the percentage of wet weight.
[c]Only FA > 1% of total FA are shown.

ing systems: an extensive system (pond, Gelucourt, France), a semiextensive system (tank, Lindre, France), and an intensive system (water recirculating system, Lucas Perches pilot farm, Hampont, France). In the extensive system, fish lived on natural prey. Fish from the semiextensive system were fed both natural food and an artificial food, Bio-Optimal ST (BioMar, Brande, Denmark). In the intensive system, fish were exclusively fed an artificial food, Ecolife 15 (BioMar) (Table 1). All fish were slaughtered by thermoshock immediately after collection. Fish were filleted 48 h post mortem, i.e., after rigor mortis (10), and the fillets were stored at −20°C awaiting lipid extraction.

Lipid extraction and FA composition. Both fillets of each fish were crushed together to homogenize the samples and to avoid any effect related to the heterogeneity of lipid distribution. Total lipids (expressed as a percentage of the fillet weight) were extracted with dichloromethane/methanol (2:1, vol/vol) according to Folch *et al.* (11) as modified by Chen *et al.* (12). BHT (0.1%) was added to the dichloromethane/methanol as an antioxidant. FA of the total lipids were converted to methyl esters with BF_3–methanol (14%) (13) and stored at −80°C. The FA composition was assessed using GLC on a fused-silica capillary column 30 × 0.25 mm i.d. and 20 µm film thickness (SUPELCOWAX™ 10; Sigma-Aldrich, St Quentin Fallavier, France). The instrument conditions were as follows: initial temperature: 140°C; heating rate: 4°C/min; final temperature: 240°C; injector temperature: 260°C; detection temperature: 260°C; carrier gas: helium; sample injection: 1 µL; total time of analysis: 26 min. FAME mixtures No. 189-19 and PUFA 3 (Sigma Chemical Company, St. Louis, MO) were used as standards. Only FA > 1% of total FA are reported in this study.

Stable isotope analysis. Diet is the primary determinant of animal isotopic composition (14). Thus, to characterize the place of perch in the trophic web and obtain some indirect information on the composition of their diet, a stable isotope analysis (^{13}C and ^{15}N) was carried out; 20 fish from the Rhine River, 20 fish from Lake Geneva (5 fish per season), and 5 fish from each rearing system were randomly selected. After lipid extraction, samples were dried for 48 h at 60°C. Isotopic ratios were determined by a coupled system of an elemental analyzer (NA 1500NC; Carlo Erba, Rodano, Italy) and a mass spectrometer (IRMS) (delta S; Finnigan, Bremen, Germany). Abundances of the ^{13}C and ^{15}N isotope were expressed in δ notation as the deviation from standards in parts per thousand (‰) according to the following equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X represents either ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The R standard values were based on PeeDee Belemnite (PDB) for ^{13}C and atmospheric N_2 for ^{15}N. The measurement errors for carbon and nitrogen were 0.20 and 0.25‰, respectively.

Statistical analysis. Data were treated with an ANOVA (unbalanced hierarchical ANOVA) using the statistical package SAS (SAS/STAT, 1989; SAS Institute Inc., Cary, NC) according to a mixed procedure and a univariate procedure. Seasonal effects were nested within site effects (site: DF of factor = 4; DF of residual = 370; season(site): DF of factor = 6; DF of residual = 370). The significance level was fixed at 5% using Bonferonni's *t*-test.

To characterize the effect of food on the muscle FA composition, a hierarchical clustering analysis using the aggregation criteria of Ward was performed using SPAD 5.5 software (SPAD, Paris, France). The $\delta^{13}C$ and $\delta^{15}N$ values were used as active variables. This data analysis allowed classes of fish to be characterized by a particular $\delta^{13}C$ or $\delta^{15}N$ value. Afterward, a factorial discriminant analysis (StatBoxPro 5.0; GrimmerSoft, Neuilly-Sur-Seine, France), using as discriminant factor the classes had previously created by the hierarchical clustering analysis, was performed on the major FA (16:0, 18:0, 16:1, 18:1, 18:2n-6, 20:4n-6, 20:5n-3, 22:6n-3) to assess whether the classification of fish realized according to the $\delta^{13}C$ or $\delta^{15}N$ values was related to the FA composition.

RESULTS

Nutritional quality and geographic source of wild perch. Overall, lipid contents of the perch fillet varied between 1.16 and 1.48% (Table 2). A significant difference in the total lipids of perch muscle was observed between fish collected from the Rhine River (1.21%) and Lake Geneva (1.43%). A difference in the weight of fish was reported between fish in these two natural systems, with bigger fish being found in the Rhine River. However, no linear relationship ($r^2 = 0.02$, $P > 0.05$, data not shown) was found between the mean total fillet lipid content and the weight of fish sampled in both natural environments at each season.

Total saturated FA (SFA) accounted for 25.5 and 25.1% of the total FA in the Rhine River and Lake Geneva fish, respectively (Table 2). Palmitic acid (16:0) was the main SFA, con-

TABLE 2
Biometric Data, Total Lipid Content (wt% of muscle), and FA Composition (% total FA) of Muscle for Wild and Farmed Eurasian Perch[a]

	Rhine	Geneva	Extensive	Semiextensive	Intensive	CV RMSE (%)
Number of fish	136	143	35	35	35	
Standard length (cm)[b]	19.7 ± 6.3	14.9 ± 1.9	17.7 ± 3.0	16.7 ± 1.7	15.7 ± 1.0	
Weight (g)[b]	264.1 ± 280.9	69.9 ± 38.2	171.2 ± 90.1	117.3 ± 38.3	97.0 ± 18.6	
Total lipid content (%)[b]	1.21 ± 0.02^{a}	1.43 ± 0.02^{b}	1.16 ± 0.04^{a}	1.26 ± 0.04^{a}	1.48 ± 0.04^{b}	19.0
FA composition[c]						
14:0	1.3 ± 0.0^{a}	1.9 ± 0.0^{c}	1.6 ± 0.1^{b}	2.1 ± 0.1^{c}	2.4 ± 0.1^{d}	25.3
16:0	19.2 ± 0.2^{b}	18.4 ± 0.1^{a}	19.9 ± 0.3^{b,c}	20.2 ± 0.3^{c}	17.7 ± 0.3^{a}	9.1
18:0	4.3 ± 0.0^{b}	4.6 ± 0.0^{c}	4.9 ± 0.1^{d}	3.6 ± 0.1^{a}	4.1 ± 0.1^{b}	12.8
Total SFA	25.5 ± 0.2^{a,b}	25.1 ± 0.2^{a}	27.8 ± 0.5^{c}	26.7 ± 0.5^{b,c}	25.1 ± 0.5^{a,b}	10.5
16:1	5.7 ± 0.2^{b}	4.3 ± 0.1^{a}	6.0 ± 0.3^{b}	5.1 ± 0.3^{c,b}	4.8 ± 0.3^{a,b}	33.9
18:1	8.0 ± 0.2^{a}	8.2 ± 0.2^{a}	10.0 ± 0.3^{b}	10.4 ± 0.3^{b,c}	11.5 ± 0.3^{c}	22.4
20:1	0.2 ± 0.0^{b}	0.1 ± 0.0^{a}	0.4 ± 0.0^{c}	1.8 ± 0.0^{d}	2.4 ± 0.0^{e}	46.1
22:1	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.7 ± 0.0^{b}	1.4 ± 0.0^{c}	89.9
Total MUFA	14.1 ± 0.3^{b}	12.5 ± 0.3^{a}	16.5 ± 0.6^{c}	17.4 ± 0.6^{c}	19.0 ± 0.6^{c}	25.0
18:2n-6	2.1 ± 0.1^{a}	3.0 ± 0.1^{b}	6.0 ± 0.1^{d}	4.6 ± 0.1^{c}	5.9 ± 0.1^{d}	21.4
20:4n-6	6.6 ± 0.1^{b}	9.5 ± 0.1^{c}	10.7 ± 0.2^{d}	1.9 ± 0.2^{a}	1.9 ± 0.2^{a}	15.7
Total n-6 PUFA	9.3 ± 0.1^{b}	12.7 ± 0.1^{c}	17.6 ± 0.3^{d}	7.0 ± 0.3^{a}	8.5 ± 0.3^{b}	14.9
18:3n-3	1.3 ± 0.2^{a}	2.5 ± 0.2^{b,c}	3.5 ± 0.3^{c}	1.6 ± 0.3^{a,b}	1.4 ± 0.3	101.6
18:4n-3	0.2 ± 0.0^{a}	0.9 ± 0.0^{c}	0.3 ± 0.0^{b}	0.4 ± 0.0^{b}	1.0 ± 0.0^{c}	51.0
20:5n-3	12.5 ± 0.1^{c}	13.2 ± 0.1^{d}	10.3 ± 0.3^{b}	9.2 ± 0.3^{a}	10.9 ± 0.3^{b}	13.1
22:6n-3	37.1 ± 0.3^{c}	31.9 ± 0.3^{b}	21.3 ± 0.7^{a}	36.9 ± 0.7^{c}	33.0 ± 0.7^{b}	11.8
Total n-3 PUFA	51.2 ± 0.3^{c}	49.0 ± 0.3^{b}	36.6 ± 0.6^{a}	48.8 ± 0.6^{b}	47.2 ± 0.6^{b}	7.9
n-3/n-6 ratio	5.9 ± 0.1^{c}	3.8 ± 0.1^{b}	2.1 ± 0.3^{a}	7.1 ± 0.3^{d}	5.8 ± 0.3^{c}	32.8

[a]RMSE, root mean square error; SFA, saturated FA; MUFA, monounsaturated FA.
[b]Values are mean ± SE.
[c]Only FA > 1% of total FA are shown. Values are adjusted mean ± SE. Values in the same row having different roman superscript letters are significantly different ($P < 0.05$).

tributing approximately 73–75% of the total SFA. The total SFA content displayed no significant difference between the two origins, although the 16:0 and 18:0 contents were significantly different.

The total monounsaturated FA (MUFA) content for fish coming from the Rhine River (14.1 ± 0.3%) was significantly higher than that from the lacustrine perch (12.5 ± 0.3%). Furthermore, no significant relationship was found between the mean MUFA content and the total lipid content ($P > 0.05$) (Fig. 1). Oleic acid (18:1) was the primary MUFA (57–66% of total MUFA) and showed no significant difference between fish from the Rhine River and Lake Geneva.

Arachidonic acid (ARA, 20:4n-6) accounted for 71 and 75% of the total n-6 PUFA in fish coming from the Rhine River and Lake Geneva, respectively (Table 2). The total n-6 PUFA content, ARA, and linoleic acid (18:2n-6) were significantly lower for fish from the Rhine River than for the lacustrine fish. Furthermore, there was a positive linear relationship between the lipid content and n-6 PUFA (Fig. 1).

DHA (22:6n-3) and EPA (20:5n-3) were identified as the main n-3 PUFA. DHA was the dominant FA in all fish, whatever their origin (between 21 and 37% of total FA) (Table 2). The n-3 PUFA content varied significantly between natural sites and was negatively related to the lipid content (Fig. 1). Fish from Lake Geneva had lower DHA and higher EPA than their counterparts from the Rhine River.

Nutritional quality and rearing systems. For each rearing system, no significant linear relationship between the weight of reared fish and the lipid content or FA was found. The rearing system had an effect on the total lipid content (Table 2). Indeed, fish from the intensive rearing system displayed a significantly higher lipid content (1.48%) than did the other farmed perch (1.26% for the semiextensive system and 1.16% for the extensive system).

The total SFA content of farmed perch varied between 25.1 and 27.8% of the total FA and was significantly lower in the intensive system. 16:0 accounted for 71–76% of the total SFA content.

No difference in the total MUFA content was noticed between the different rearing systems. Oleic acid was dominant

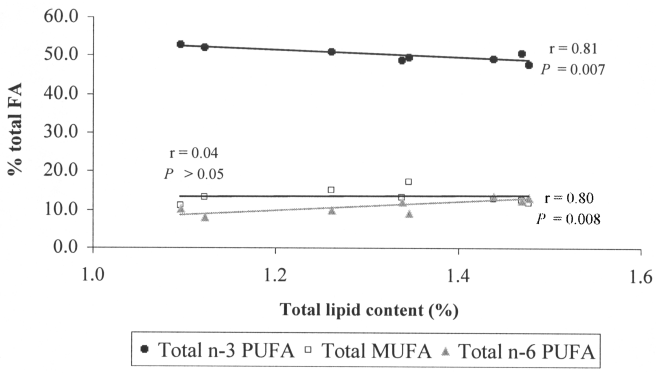

FIG. 1. Relationship between the total lipid content (%) and the monounsaturated FA (MUFA), n-3 PUFA, and n-6 PUFA contents of fish (% of total FA). Values represented are the average total lipid content and MUFA, n-3 PUFA, or n-6 PUFA content of fish in each natural system and season.

TABLE 3
Biometric Data, Seasonal Total Lipid Content (wt% of muscle), and FA Composition (% total FA) of Muscle for Wild Eurasian Perch Sampled from the Rhine River

	July 2002	October 2002	January 2003	April 2003	CV RMSE (%)
Number of fish	38	35	39	24	
Standard length (cm)[a]	14.0 ± 3.3	18.2 ± 5.3	25.6 ± 4.6	21.1 ± 4.7	
Weight (g)[a]	73.2 ± 75.3	184.5 ± 176.7	526.2 ± 322.0	272.5 ± 245.6	
Total lipid content (%)[a]	1.12 ± 0.04[a]	1.10 ± 0.04[a]	1.35 ± 0.05[b]	1.26 ± 0.04[a,b]	19.0
FA composition[b]					
14:0	1.3 ± 0.1[a]	1.1 ± 0.1[a]	1.4 ± 0.1[a]	1.4 ± 0.1[a]	25.3
16:0	19.5 ± 0.3[a]	19.4 ± 0.3[a]	19.0 ± 0.3[a]	18.8 ± 0.4[a]	9.1
18:0	5.5 ± 0.1[c]	4.8 ± 0.1[b]	3.4 ± 0.1[a]	3.6 ± 0.1[a]	12.8
Total SFA	27.2 ± 0.4[c]	26.2 ± 0.5[b,c]	24.1 ± 0.44[a]	24.4 ± 0.5[a,b]	10.5
16:1	5.1 ± 0.3[a,b]	4.2 ± 0.3[a]	7.7 ± 0.3[c]	6.0 ± 0.4[b]	33.9
18:1	7.6 ± 0.3[a,b]	6.6 ± 0.3[a]	9.4 ± 0.3[c]	8.5 ± 0.4[b,c]	22.4
Total MUFA	13.1 ± 0.6[a,b]	11.0 ± 0.6[a]	17.4 ± 0.6[c]	14.9 ± 0.7[b,c]	25.0
18:2n-6	1.7 ± 0.1[a]	2.1 ± 0.1[a,b]	2.2 ± 0.1[a,b]	2.5 ± 0.1[b]	21.4
20:4n-6	5.8 ± 0.2[a]	7.1 ± 0.2[b]	6.5 ± 0.2[a,b]	6.9 ± 0.2[b]	15.7
Total n-6 PUFA	7.9 ± 0.3[a]	10.1 ± 0.3[b]	9.2 ± 0.3[a,b]	9.8 ± 0.3[b]	14.9
18:3n-3	1.0 ± 0.3[a]	1.3 ± 0.3[a]	2.0 ± 0.3[a]	0.8 ± 0.4[a]	101.6
20:5n-3	14.0 ± 0.3[b]	12.4 ± 0.3[a]	11.7 ± 0.3[a]	11.7 ± 0.3[a]	13.1
22:6n-3	36.3 ± 0.6[a]	38.4 ± 0.7[a]	35.7 ± 0.6[a]	38.3 ± 0.8[a]	11.8
Total n-3 PUFA	51.8 ± 0.6[a,b]	52.6 ± 0.6[b]	49.4 ± 0.6[a]	50.9 ± 0.8[a,b]	7.9
n-3/n-6 ratio	7.1 ± 0.3[b]	5.5 ± 0.3[a]	5.6 ± 0.3[a]	5.5 ± 0.3[a]	32.8

[a]Values are mean ± SE.
[b]Only FA > 1% of total FA are shown. Values are adjusted mean ± SE. Values in the same row having different roman superscript letters are significantly different ($P < 0.05$). For abbreviations see Table 2.

and increased significantly as the level of farming intensified.

The n-6 PUFA contents exhibited a significant difference between the rearing systems (Table 2). The lowest total n-6 PUFA level was found in fish from the semiextensive system (7.0% of total FA), whereas fish from the extensive system had a higher n-6 PUFA content (17.6% of total FA).

Concerning the n-3 PUFA, the lowest values were found in fish from the extensive system, whereas the highest values were assessed in fish from the semiextensive and intensive systems (Table 2). The semiextensively farmed fish showed a higher proportion of DHA (36.9%) and a lower level of EPA (9.2%) in comparison with the other farmed fish.

Comparison between wild and farmed perch. The total lipid content in farmed perch showed significant differences from wild fish, but these differences depended on the rearing system and the natural system considered (Table 2). Two different groups could be formed according to their similar lipid content, namely, (i) fish from the Rhine River and from the extensive and semiextensive systems, and (ii) fish sampled from Lake Geneva and from the intensive system.

The most important differences in FA composition between the wild and farmed fish were in their MUFA and PUFA contents (Table 2). The farmed fish had a higher MUFA content than did wild fish (16–19% vs. 12–14% of total FA). This was particularly obvious for oleic acid (10–11.5% vs. 8–8.2% of total FA). ARA was identified as the primary n-6 PUFA in fish coming from the Rhine River, Lake Geneva, and the extensive system (71, 75, and 61% of total n-6 PUFA respectively), i.e., fish fed with natural food, whereas linoleic acid was predominant in fish reared in the semiextensive and intensive systems (66 and 69% of n-6 PUFA, respectively). The wild fish displayed significantly more EPA (13.2–12.5% of total FA) than the farmed perch (9.2–10.9%), whereas no difference was found for DHA between (i) the lacustrine and intensively farmed fish, and (ii) the Rhine River and semiextensively farmed fish.

Seasonality of the nutritional quality in wild perch fillets. The season had a significant effect on the total lipid content of fish from the Rhine River (Table 3), whereas no effect was detected for perch sampled from Lake Geneva (Table 4). A slightly positive linear relationship was reported between the mean lipid content and the mean weight of fish by season for the Rhine River ($r^2 = 0.83$, $P = 0.046$, data not shown), but not in fish from Lake Geneva ($r^2 = 0.61$, $P > 0.05$, data not shown), probably because of their low weight heterogeneity.

Seasonal variability in the FA composition was greater for fish sampled from the Rhine River, probably because of the high size heterogeneity compared with those sampled from Lake Geneva (Tables 3, 4). In this study, the proportion of 16:0 remained constant throughout the year. However, the proportion of SFA exhibited some variability, with the lowest value in January and the highest in July.

Concerning the total MUFA content, no seasonal variation appeared in lacustrine fish, whereas the MUFA content of Rhine River fish reached a minimum in October 2002 (11% of total FA) and a maximum in January 2003 (17.4% of total FA). Moreover, a positive relationship ($r^2 = 0.94$, $P = 0.015$, data not shown) was found between the total lipid content and the MUFA content of fish sampled from the Rhine River.

The n-6 PUFA content varied significantly between seasons

TABLE 4
Biometric Data, Seasonal Total Lipid Content (wt% of muscle), and FA Composition (% total FA) of Muscle for Wild Eurasian Perch Sampled from Lake Geneva

	July 2002	October 2002	January 2003	April 2003	CV RMSE (%)
Number of fish	34	29	48	32	
Standard length (cm)[a]	13.8 ± 3.1	14.8 ± 0.9	15.2 ± 1.0	15.5 ± 1.6	
Weight (g)[a]	60.5 ± 65.1	74.2 ± 16.1	69.4 ± 17.6	77.0 ± 36.3	
Total lipid content (%)[a]	1.48 ± 0.04[a]	1.44 ± 0.05[a]	1.47 ± 0.04[a]	1.34 ± 0.04[a]	19.0
FA composition[b]					
14:0	2.2 ± 0.1[b]	1.9 ± 0.1[b]	1.5 ± 0.1[a]	1.9 ± 0.1[b]	25.3
16:0	18.7 ± 0.3[a]	17.6 ± 0.3[a]	18.5 ± 0.3[a]	18.9 ± 0.3[a]	9.1
18:0	5.8 ± 0.1[c]	4.7 ± 0.1[b]	4.1 ± 0.1[a]	3.9 ± 0.1[a]	12.8
Total SFA	26.7 ± 0.5[b]	24.6 ± 0.5[a,b]	24.4 ± 0.4[a]	24.6 ± 0.5[a,b]	10.5
16:1	3.7 ± 0.3[a]	4.3 ± 0.3[a]	4.2 ± 0.3[a]	4.9 ± 0.3[a]	33.9
18:1	7.6 ± 0.3[a]	8.5 ± 0.4[a]	8.2 ± 0.3[a]	8.5 ± 0.4[a]	22.4
Total MUFA	11.8 ± 0.6[a]	12.8 ± 0.7[a]	12.3 ± 0.5[a]	13.1 ± 0.6[a]	25.0
18:2n-6	3.2 ± 0.1[b]	2.9 ± 0.1[a,b]	2.7 ± 0.1[a]	3.0 ± 0.1[a,b]	21.4
20:4n-6	9.5 ± 0.2[a]	9.6 ± 0.2[a]	9.7 ± 0.2[a]	9.2 ± 0.2[a]	15.7
Total n-6 PUFA	13.2 ± 0.3[a,b]	13.4 ± 0.3[b]	12.4 ± 0.2[a,b]	12.0 ± 0.3[a]	14.9
18:3n-3	2.6 ± 0.3[a]	2.9 ± 0.4[a]	2.1 ± 0.3[a]	2.3 ± 0.4[a]	101.6
18:4n-3	1.3 ± 0.0[b]	1.1 ± 0.1[b]	0.6 ± 0.0[a]	0.7 ± 0.0[a]	51.0
20:5n-3	16.3 ± 0.3[c]	14.0 ± 0.3[b]	11.8 ± 0.2[a]	10.7 ± 0.3[a]	13.1
22:6n-3	26.8 ± 0.7[a]	30.6 ± 0.7[b]	35.7 ± 0.6[c]	34.5 ± 0.7[c]	11.8
Total n-3 PUFA	47.4 ± 0.7[a]	49.2 ± 0.7[a,b]	50.6 ± 0.6[b]	48.8 ± 0.7[a,b]	7.9
n-3/n-6 ratio	3.6 ± 0.3[a]	3.7 ± 0.3[a]	4.1 ± 0.2[a]	4.0 ± 0.3[a]	32.8

[a]Values are mean ± SE. For abbreviations see Table 2.
[b]Only FA > 1% of total FA are shown. Values are adjusted mean ± SE. Values in the same row having different roman superscript letters are significantly different ($P < 0.05$).

in both sites. ARA showed no seasonal variation for the lacustrine fish (9.2–9.7% of total FA), whereas the proportion in fish from the Rhine River was significantly higher in October and April (7.1 and 6.9% of total FA, respectively) than in July (5.8% of total FA). Moreover, no significant linear relationship was found between the n-6 PUFA content and total lipids at both sites ($P > 0.05$, results not shown).

The proportion of DHA in fish from the Rhine River remained constant throughout the year, whereas the EPA level was higher in July. However, in the lacustrine system, perch had a significantly lower proportion of DHA in July than in January and April, whereas the proportion of EPA displayed the reverse tendency.

Relationship between fish diet composition and nutritional quality. No significant relationship ($P > 0.05$) was established between the size of fish and the $\delta^{13}C$ or $\delta^{15}N$ values in both wild systems. The stable isotopic analyses clearly showed a difference in the $\delta^{13}C$ or $\delta^{15}N$ between fish from each system (reared vs. wild systems) (Fig. 2). This was confirmed by the hierarchical clustering representation, which showed five classes (Fig. 3). Classes 1 and 2 contained fish from the Rhine River. Classes 3 and 4 corresponded to fish sampled from Lake Geneva and the extensive system, respectively. Class 5 was composed of fish farmed in the intensive and semiextensive systems, i.e., fish that had been fed artificial food. Furthermore, results of the factorial discriminant analysis clearly showed that these five classes formed with hierarchical clustering analysis were successfully identified with the FA composition of muscle (96% of total variability explained by planes 1–2) (Fig. 4A). The discrimination according to these five classes, i.e., fish from the different aquatic systems (wild and reared), was mainly realized according to the ARA, DHA, and 18:2n-6 contents of fillets (Fig. 4B). Moreover, it clearly showed that the FA composition of perch reared in the semiextensive and intensive systems was highly correlated with the diet composition (Tables 1, 2). Thus, all of these results suggest that differences in the nutritional quality of fish from different aquatic systems (wild or reared) were mainly the result of different diets.

DISCUSSION

Variability of total lipid content according to the geographic source and rearing system. The lipid contents in perch fillets measured in the present study are in agreement with the litera-

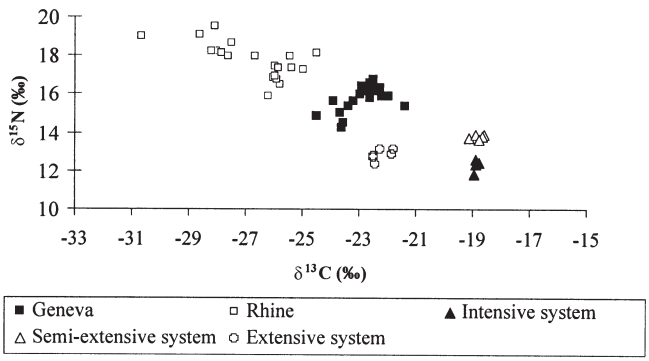

FIG. 2. Stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) of fish fillets in natural and rearing systems.

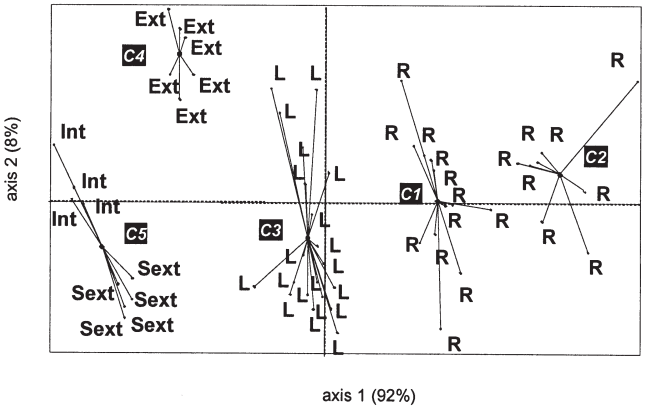

FIG. 3. Hierarchical clustering representation for stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) of fish in natural and rearing systems. C1, 2, 3, 4, and 5 = class 1, 2, 3, 4, and 5; R = fish from the Rhine River; L = fish from Lake Geneva; Ext = fish from the extensive system; Sext = fish from the semiextensive system; Int = fish from the intensive system.

ture (7,15). The lipid content was variable, but the range of variation was low. The higher lipid content found in Lake Geneva fish could be related to their diet or to a lower annual water temperature compared with the Rhine River (10.5 vs. 16°C, respectively) (9). Indeed, according to Corraze *et al.* (16), the increase in lipid content at low temperatures could be the result of a greater deposit of dietary lipids. But other factors (such as genetics and lipid metabolism) also could be implicated in the lipid content in muscle, as suggested by Henderson and Tocher (17).

The higher lipid content in fish from the intensive system was related to the dietary lipid content (Table 1). However, Xu *et al.* (18) showed that an increase in fat content of the diet (11.7; 15; 19.3%) had no consequence on the lipid content of the muscle of perch, as was also found by Mathis *et al.* (15). In our study, the lipid contents of the diets for the intensively and semiextensively farmed perch (17.0 and 10.0%, respectively) were similar to those tested by Xu *et al.* (18) and Mathis *et al.* (15), but an effect on intramuscular lipid content was highlighted. Furthermore, according to Gardeur (unpublished data), among the 12 different dietary and environmental factors tested, only one interaction, i.e., between water temperature and dietary lipid sources (fish oil or rapeseed oil), led to significant differences in the muscle lipid content of intensively reared perch.

Fish from the intensive system are domesticated fish (sixth generation, or F6), with the origin of genitors (F0) being Neuchatel Lake (Switzerland). Thus, the close geographic origin of fish from Lake Geneva and fish used in the intensive rearing system could suggest that the fish actually belong to the same population, explaining their similar lipids content.

Moreover, even if the amount of food consumed had not been determined in our experiment, this factor could be implicated in the difference in fillet lipid contents reported here. Indeed, this factor was important in determining the lipid content in *Dicentrarchus labrax* (19). But according to Mathis *et al.* (10), no effect of feeding rate on the total lipid content of perch

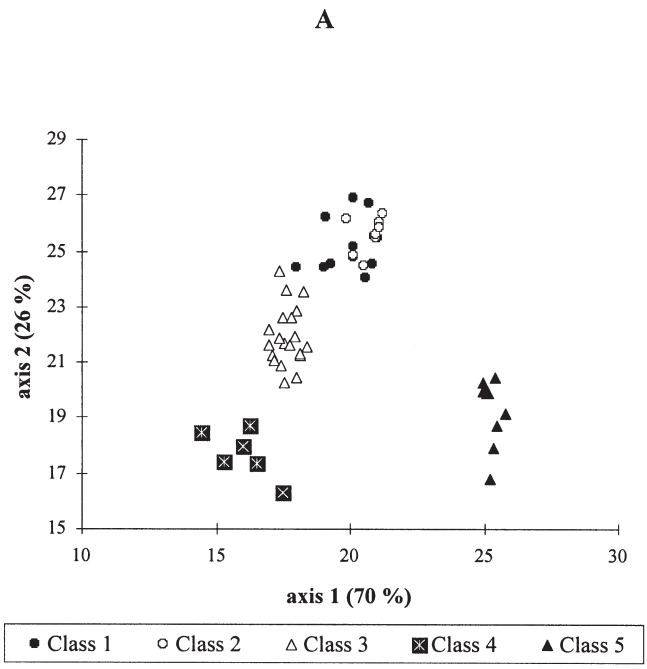

FIG. 4. 2-D plot of the FA profiles of muscle in fish sampled in all systems with a factorial discriminant analysis using classes derived from the hierarchical clustering representation as the discriminant factor. (A) Results of the factorial discriminant analysis for FA variables; (B) results of the factorial discriminant analysis for individuals.

fillets was found in intensive rearing conditions.

Variability of the FA composition according to the geographic source and rearing system. The total SFA content reported here is in agreement with other studies in which SFA represent less than 30% of the total FA (20). Moreover, the 16:0 content (73–75% of the total SFA content) was slightly lower than those reported by Xu and Kestemont (7) and Xu *et al.* (18)

in perch (78–82%), but higher than those reported in the sander *Sander lucioperca* (65%) (21).

The difference in the MUFA content between fish from the Rhine River and Lake Geneva was not related to the lipid content but might be explained by the difference in the dietary FA composition of wild perch. For both wild and farmed fish, particularly salmonids, it is well established that the FA composition of fillets reflects the composition of the fish diet (22). The stable isotope analysis tended to support this pattern: A difference in the diet composition might have been responsible for the difference in the FA composition of fish coming from each system (natural and farmed).

In this study, the n-3 PUFA content was negatively related to the total lipid content. When the total lipid content increases, TAG are accumulated, whereas the cellular phospholipids, which are rich in n-3 PUFA and particularly in DHA, remain constant (23,24). Therefore, phospholipids and DHA decrease proportionally with a higher lipid content, as reported in the present study.

The higher ARA values in wild fish and in fish from the extensive system compared with farmed fish are consistent with findings from other researchers (25,26) and are related to the composition of their natural prey (27). Furthermore, the composition of the commercial food available in the semiextensive and intensive systems, which showed a high proportion of linoleic acid and a low level of ARA, explains the fillet FA composition of the farmed perch. Moreover, the higher linoleic acid content found in the extensive system could suggest a diet composition rich in this FA.

A higher DHA content in wild fish, in comparison with the farmed counterparts, has already been described (4,20,26). However, the opposite results were found in the gilthead sea bream *Sparus aurata* (28), although for the same species, Grigorakis *et al.* (25) found no difference, as was also found in the white seabream (29). Effects of geographic origin, environment, and strain could be important in the FA composition of wild and reared fish.

In freshwater fish, the n-3/n-6 ratios range from 1 to about 4 (30). In perch filet, our results showed that this ratio varied according to the source but always ranged at the higher level (5.9 ± 0.1%, 7.1 ± 0.3%, 5.8 ± 0.1% for fish from the Rhine River and from the semiextensive and intensive rearing systems, respectively). These high values impart to perch a good nutritional value among all the freshwater fish. Differences in n-3/n-6 ratios existed between wild and farmed fish. Indeed, some authors have found a higher n-3/n-6 ratio in wild fish than in farmed counterparts (20,25,26,29), but the amplitude of these differences between wild and farmed fish depended on the dietary FA composition. Indeed, the level of n-3 PUFA in the diet could vary greatly according to the dietary lipid source and *in fine* to induced variations in the n-3/n-6 ratio in fillets (31,32). However, the overall nutritional quality of perch depended both on the rearing system and on the geographic source of the fish, as well as on the important lipid classes.

In this study, fish from natural systems and from the extensive system consumed natural prey, whereas fish from the intensive system were exclusively fed an artificial food. In the semiextensive system, fish could eat both natural and artificial food. The lack of similarity in the FA profiles between fish from the natural system and from the extensive rearing system still remains unclear. The stable isotope analysis showed that extensively reared fish possessed stable isotope values different from fish from the Rhine River and Lake Geneva. This suggests a different diet composition between fish from natural and extensive systems, e.g., a lower level of n-3 PUFA and a higher level of n-6 PUFA, particularly in linoleic acid, for prey from the extensive system compared with those from the Rhine River and Lake Geneva. However, factors other than food could be important, such as environmental conditions, as suggested by Njinkoue *et al*. (33), or some intrinsic factor (strain or lipid metabolism).

Effect of season on the total lipid content and FA composition of wild perch. The seasonal variation in lipid content primarily affects the tissue where fat stores are preferentially accumulated (34,35). Perch deposit lipids in the perivisceral tissue rather than in the muscle (18). This suggests that the muscle lipid content would remain constant throughout the year, as reported in this study, particularly for fish sampled from Lake Geneva. The higher weight of fish collected in the Rhine River in January might explain the higher total lipid content found in this period, because of the linear relationship between the weight of fish from the Rhine River and the lipid content. It thus appears that in this study, the apparent seasonality of the total lipid content in fillets of fish from the Rhine River is size related, although other factors, such as temperature or diet composition (33), could explain this variation.

The seasonal variations in FA composition also have been described as species dependent or FA dependent (36). For example, significant differences between the seasons were reported in the FA composition of the common sole *Solea solea* (35), in the sardine *Sardinops melanostictus* (37), in the Japanese catfish, *Silurus asotus* (38), and in the wild crappie, *Pomoxis* spp. (39). In our study, the relationship between the MUFA content and lipid content of fish was previously found by Mathis *et al.* (40). Consequently, for fish from the Rhine River, the increase in lipid content in January induced a higher proportion of MUFA, thereby increasing the relative proportion of MUFA, presumably in TG.

In perch coming from the Rhine River, the proportion of EPA varied according to the season, whereas the DHA content remained almost constant irrespective of the season. However, for lacustrine perch, the seasonal variation in EPA (low in July, i.e., end of the spawning period; high in January and April) and DHA (high in July) could suggest a selective mobilization of EPA and/or a specific retention of DHA during gonad development, as has been found in the European whitefish *Coregonus macrophthalmus* (41). Other hypotheses could be suggested, such as quantitative or qualitative changes in the diet composition of perch between the summer and winter, as was observed for the sardine *Sardinops melanostictus* (37). However, in the present study, the stable isotope analysis was not sufficient to decisively link seasonal variation in the FA composition with

variation in the diet composition. Indeed, it has been reported in the bivalve *Pecten maximus* that seasonal variations in metabolism can cause changes in the isotopic composition unrelated to changes in the diet (14). A gut content analysis could give complementary data, allowing us to study whether the seasonality of the fillet FA composition is linked to variations in the prey consumed.

The aim of this work was to compare the nutritional quality of perch fillets coming from different sources (wild, reared) that are available on the market. We showed that the nutritional quality of the perch varied according to the geographic source, rearing system, and season. All these variations were mainly correlated with the diet composition of the fish. However, the high n-3 PUFA content and n-3/n-6 ratio identify this species as a product of high nutritional quality. Further experimental studies should be developed to assess, independently of diet, the true effect of the season, geographic source, and rearing system.

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Fatty Acid Profiles and Relative Mobilization During Fasting in Adipose Tissue Depots of the American Marten (*Martes americana*)

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ABSTRACT: The American marten (*Martes americana*) is a boreal forest marten with low body adiposity but high metabolic rate. The study describes the FA composition in white adipose tissue depots of the species and the influence of food deprivation on them. American marten ($n = 8$) were fasted for 2 d with 7 control animals. Fasting resulted in a 13.4% weight loss, while the relative fat mass was >25% lower in the fasted animals. The FA composition of the fat depots of the trunk was quite similar to other previously studied mustelids with 14:0, 16:0, 18:0, 16:1n-7, 18:1n-9, and 18:2n-6 as the most abundant FA. In the extremities, there were higher proportions of monounsaturated FA (MUFA) and PUFA. Food deprivation decreased the proportions of 16:0 and 16:1n-7, while the proportion of long-chain MUFA increased in the trunk. The mobilization of FA was selective, as 16:1n-7, 18:1n-9, and particular n-3 PUFA were preferentially mobilized. Relative mobilization correlated negatively with the carbon chain length in saturated FA (SFA) and n-9 MUFA. The Δ9-desaturation of SFA enhanced the mobilization of the corresponding MUFA, but the positional isomerism of the first double bond did not correlate consistently with relative mobilization in MUFA or PUFA. In the marten, the FA composition of the extremities was highly resistant to fasting, and the tail tip and the paws contained more long-chain PUFA to prevent the solidification of lipids and to maintain cell membrane fluidity during cooling.

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The American marten (*Martes americana*) is a boreal forest marten, together with the Japanese marten (*M. melampus*), the sable (*M. zibellina*), and the European marten (*M. martes*; 1,2). The American marten occupies a large part of North America (3), inhabiting forests or landscape mosaics with seasonal snow cover (4). The species prefers loose snow with access to subnivean spaces, which has been interpreted as a strategy to minimize energy loss (5). Metabolic costs are a major factor determining the seasonal habitat choice for the American marten. This could be due to the high energetic costs associated with the elongated body shape (6–8). The most important food items in the diet of the American marten are voles, birds, fish, ungulate carcasses, insects, fruits, and berries (9).

The American marten has a low body fat content (approximately 5%) possibly as an adaptation to maintain the lean body shape required for hunting in the burrows of rodents (10). Furthermore, the species does not experience clear seasonal cycles in its body fat content (11). Due to its small gastric ventricle, the American marten has only a limited capacity to consume large amounts of food during a single meal, making fat deposition difficult. The high-protein diet and the high energetic costs of foraging are additional causes for the limited fat storage capacity. However, it has been established that the species can withstand at least 5 d of fasting by entering a state of protein conservation using fat and protein in a ratio of 1.56:1 (12). The plasma concentrations of particular amino acids increase and the excretion of urea decreases in fasted marten probably owing to a high turnover of proteins and urea nitrogen recycling (13). These results indicate that the American marten must be able to mobilize some of its fat reserves effectively for short periods of time. In fact, the periods of naturally occurring fasting are relatively short for the marten, as they are also known to leave their nests daily during the winter with the exception of the harshest weather conditions (14).

In addition to fur, the subcutaneous (sc) fat layer may offer thermal insulation for the marten in the boreal climate. There must also exist a temperature gradient from the skin to the core of the body affecting the FA composition. The challenge of the ambient temperature is even more pronounced on the extremities, such as on the bare food pads and the tip of the tail. Unlike the sc or intra-abdominal (iab) fat depots, the fats of the footpads have to maintain cell membrane fluidity, and it can be hypothesized that they would experience only minor changes in FA composition during food scarcity. For instance, iab fat depots of the semiaquatic American mink (*Mustela vison*) are preferentially mobilized instead of sc fat probably owing to the significance of the sc fat layer as insulation during aquatic predation (15; Nieminen, P., Käkelä, R., Pyykönen, T., and Mustonen, A.-M., unpublished data).

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Abbreviations: BM, body mass; BMI, body mass index; DBI, double bond index; DI, desaturation index; dia, diaphragmatic; iab, intra-abdominal; mes, mesenteric; MUFA, monounsaturated FA; om, omental; rp, retroperitoneal; sc, subcutaneous; SFA, saturated FA; TACL, total average chain length; UFA, unsaturated FA; VLCFA, very long chain FA; WAT, white adipose tissue.

The aim of this study was to describe (i) the FA composition of different white adipose tissue (WAT) depots of the American marten and (ii) the effects of a 48-h fasting period on FA composition. The fasting period of 2 d was considered suitable because, according to previous studies (12), it is significantly shorter than what the species can withstand.

EXPERIMENTAL PROCEDURES

For these experiments 15 American marten (9 males and 6 females) born between 1993 and 2004 were randomly divided into two experimental groups. All the animals were housed singly in standard cages (85 × 30 × 46 cm) with wooden nest boxes (24 × 32 × 35 cm) suspended above ground in an unheated shed at the Canadian Centre for Fur Animal Research, Nova Scotia Agricultural College, in Truro, Nova Scotia, Canada (45.37°N, 63.27°W). Before the experiment the animals were fed for several months with standard fur animal feed (metabolizable energy 1200–1300 kcal kg fresh wt^{-1}; protein minimum 11.0–12.0%, fat 3.0–7.5%, carbohydrates maximum 10.0–11.0%; 16). The marten were provided about 200 g feed or 250 kcal $animal^{-1}$ d^{-1}. They are known to require about 80 kcal d^{-1} when at rest (17). The fasting experiments were conducted January 5–7, 2005. Half of the animals (n = 8) were put on a total 2-d fast, while the other half (n = 7) was fed using the diet and procedure described above. The fed group was fasted overnight before the sampling. Water was available for both experimental groups *ad libitum* during the whole of the study period.

Body masses (BM) were recorded on the first day of the experiment and at sampling, and the body lengths at sampling. Body mass indices (BMI) indicating body adiposity of mustelids (15) were calculated with the formula BMI = BM (kg) × [body $length^3$ (m)]$^{-1}$. The animals were anesthetized with an intramuscular injection of xylazine (Rompun™ 0.17 mL kg^{-1}) and ketamine hydrochloride (Ketalean™ 0.09 mL kg^{-1}). Blood samples were obtained by cardiac punctures using sterile evacuated blood collection tubes with EDTA as an anticoagulant, and the animals were euthanized with an intracardiac injection of pentobarbital (Euthanyl™ 0.44 mL kg^{-1}). The blood was stored on ice until centrifugation at 1000 × g for 15 min at 4°C, after which the plasma was removed. The livers and WAT samples were dissected. The WAT samples collected were as follows: sc interscapular, sc rump, sc ventral, iab omental (om), iab mesenteric (mes), iab diaphragmatic (dia), iab retroperitoneal (rp), intermuscular, tail tip, front paw, and rear paw. The fat samples of the paws were obtained from the tissues situated ventral to the metacarpal and metatarsal bones. At the same time, muscle and kidney samples were obtained for other substudies, and the spleen and pancreas were weighed. All the samples were placed into vials, frozen immediately with liquid nitrogen, and stored at −80°C. The carcasses were also frozen and the different adipose depots dissected and weighed at a later date.

The FA compositions of WAT, plasma, and liver were determined from total lipids. Subsamples of WAT and liver were transmethylated according to Christie (18) by heating with 1% methanolic H_2SO_4 under a nitrogen atmosphere. The same procedure was used for the plasma samples (50 μL); these were first evaporated to near dryness under nitrogen flow and then methylation solvents were added immediately. The FAME that formed were extracted with hexane. The dried and concentrated FAME were analyzed by a gas–liquid chromatograph (GC-FID and GC-MS, 6890N network GC system with autosampler, FID detector, and model 5973 mass selective detector; Agilent Technologies Inc., Palo Alto, CA). The injection volume was 2 μL, and the split ratio 1:20. The injectors were set at 250°C, and the FID and mass interphases were at 250 and 200°C, respectively. Helium was used as a carrier gas (1.8 and 1.0 mL min^{-1} for FID and mass detecting lines, respectively). The obtained peaks were reintegrated manually. The FAME were identified based on retention time, MS, and comparisons with authentic (Sigma, St. Louis, MO) and natural standards of known composition and published reference spectra (http://www.lipidlibrary.co.uk/masspec.html). Quantifications were based on FID responses. The peak areas of the FID chromatograms were converted to mole percentages by using the theoretical response factors (19) and calibrations with quantitative authentic standards. The FA were marked by using the abbreviations: [carbon number]:[number of double bonds] n-[position of the first double bond calculated from the methyl end] (e.g., 22:6n-3). The iso- and anteiso-isomers were abbreviated as *i* and *ai*. If not stated otherwise, PUFA were methylene-interrupted. The FA results are represented as mol%.

The double bond index (DBI) and the total average chain length (TACL), indicating the mean number of double bonds and the mean number of carbon atoms per molecule, respectively, were calculated according to standard formulae (20). The Δ9-desaturation index (Δ9-DI), the ratio of the most important potentially endogenous Δ9-monounsaturated FA (MUFA) to the corresponding saturated FA (SFA), was calculated as [(mol% 14:1n-5) + (mol% 16:1n-7) + (mol% 16:1n-9) + (mol% 18:1n-9) + (mol% 18:1n-7)]/[(mol% 14:0) + (mol% 16:0) + (mol% 18:0)]. The very long chain FA (VLCFA) were calculated as the sum of all FA with a chain length ≥24 carbons (i.e., 24:0 + 24:1n-9; Ref. 21). Relative mobilization was calculated by the formula: [(mol% in the fed animals) – (mol% in the fasted animals)]/(mol% in the fed animals). The FA composition and relative mobilization were also determined for the pooled sc (scapular, rump, and ventral combined) and iab (om, mes, rp and dia combined) fat depots and compared with the results obtained from the extremities.

Multiple comparisons between the experimental groups were performed with one-way ANOVA followed by the Duncan's *post hoc* test. Comparisons between the two experimental groups were performed with the Student's *t*-test for independent samples or, for nonparametric data, with the Mann–Whitney U test. Correlations were calculated using the Spearman correlation coefficient (r_s). A P value less than 0.05 was considered to be statistically significant. The results are presented as mean ± SE.

TABLE 1
Effects of Fasting on the FA (mol%) in Total sc and iab Fats, Fats of the Extremities, and Plasma and Liver of the American Marten[a] (mean ± SE)

	Total sc fat		Total iab fat		Tail tip		Front paws		Rear paws		Plasma		Liver	
FA	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
12:0	1.40 ± 0.10[b]	1.27 ± 0.09	1.28 ± 0.09[b]	1.18 ± 0.07	0.64 ± 0.07[a]	0.70 ± 0.03	0.85 ± 0.07[a]	1.09 ± 0.08	0.68 ± 0.12[a]	0.88 ± 0.07	0.53 ± 0.15	1.10 ± 0.28	0.10 ± 0.03*	0.76 ± 0.27*
*i*14:0	0.04 ± < 0.01*[a]	0.09 ± 0.02*	0.04 ± < 0.01*[a]	0.08 ± 0.01*	0.12 ± 0.02[b]	0.21 ± 0.10	0.07 ± 0.01[a,b]	0.06 ± < 0.01	0.06 ± 0.01[a,b]	0.06 ± 0.01	0.08 ± 0.01*	0.24 ± 0.05*	0.02 ± < 0.01	0.02 ± < 0.01
14:0	7.86 ± 0.44*[b]	6.77 ± 0.56*	7.17 ± 0.39[b]	6.11 ± 0.42	4.42 ± 0.40[a]	4.92 ± 0.22	4.71 ± 0.28[a]	5.42 ± 0.39	3.93 ± 0.36[a]	4.69 ± 0.29	0.69 ± 0.18*	1.68 ± 0.40*	0.50 ± 0.03*	1.67 ± 0.53*
*i*15:0	0.11 ± 0.01*[b]	0.18 ± 0.03*	0.11 ± 0.01*[b]	0.14 ± 0.01*	0.11 ± 0.01[b]	0.26 ± 0.14	0.08 ± 0.01[a]	0.08 ± < 0.01	0.07 ± 0.01[a]	0.08 ± 0.01	0.16 ± 0.05*	0.31 ± 0.05*	0.02 ± < 0.01*	0.04 ± 0.01*
*ai*15:0	0.06 ± < 0.01*	0.13 ± 0.02*	0.06 ± < 0.01*	0.10 ± 0.01*	0.07 ± 0.01	0.26 ± 0.18	0.07 ± 0.01	0.06 ± < 0.01	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0.02*	0.13 ± 0.04*	0.02 ± < 0.01	0.03 ± 0.01
15:0	0.31 ± 0.01[b]	0.30 ± 0.03	0.30 ± 0.01*[b]	0.25 ± 0.01*	0.26 ± 0.01[a]	0.28 ± 0.03	0.29 ± 0.02[a,b]	0.24 ± 0.01	0.26 ± 0.01[a]	0.24 ± 0.01	0.23 ± 0.04	0.29 ± 0.05	0.21 ± 0.02	0.18 ± 0.02
*i*16:0	0.06 ± < 0.01*[a]	0.13 ± 0.03*	0.05 ± < 0.01*[a]	0.08 ± 0.01*	0.11 ± 0.02[b]	0.16 ± 0.04	0.08 ± 0.01[a,b]	0.07 ± < 0.01	0.07 ± 0.01[a,b]	0.06 ± 0.01	0.10 ± 0.02	0.18 ± 0.04	0.06 ± 0.01*	0.03 ± < 0.01*
16:0	20.05 ± 0.30*[c]	15.03 ± 0.58*	20.49 ± 0.16*[c]	15.79 ± 0.52*	12.42 ± 0.61[a]	13.17 ± 0.76	15.63 ± 0.67[b]	14.07 ± 0.42	15.41 ± 0.56[b]	14.57 ± 0.31	26.34 ± 3.52	33.01 ± 5.22	24.15 ± 1.28	23.22 ± 1.34
*i*17:0	0.13 ± < 0.01[a]	0.19 ± 0.03	0.13 ± < 0.01[a]	0.14 ± 0.01	0.18 ± 0.02[b]	0.23 ± 0.05	0.14 ± 0.01[a]	0.12 ± 0.01	0.13 ± 0.01[a]	0.12 ± 0.01	0.32 ± 0.06	0.31 ± 0.05	0.28 ± 0.02*	0.17 ± 0.03*
*ai*17:0	0.14 ± < 0.01*[a]	0.25 ± 0.04*	0.14 ± < 0.01*[a]	0.19 ± 0.02*	0.19 ± 0.01[b]	0.23 ± 0.04	0.18 ± 0.01[b]	0.18 ± 0.01	0.17 ± 0.02[a,b]	0.17 ± 0.01	0.11 ± 0.03	0.20 ± 0.04	0.12 ± 0.02	0.15 ± 0.02
17:0	0.39 ± 0.01[c]	0.43 ± 0.04	0.41 ± 0.01[c]	0.43 ± 0.02	0.25 ± 0.02[a]	0.26 ± 0.02	0.33 ± 0.02[b]	0.29 ± 0.02	0.32 ± 0.02[b]	0.27 ± 0.01	0.77 ± 0.11	0.82 ± 0.12	0.91 ± 0.06*	0.65 ± 0.07*
*i*18:0	0.08 ± < 0.01[a]	0.13 ± 0.02	0.07 ± < 0.01[a]	0.12 ± 0.02	0.11 ± 0.01[b]	0.16 ± 0.03	0.08 ± 0.01[a]	0.08 ± 0.01	0.06 ± 0.01[a]	0.09 ± 0.02	0.11 ± 0.04	0.17 ± 0.03	0.06 ± < 0.01*	0.04 ± < 0.01*
18:0	5.72 ± 0.15*[a]	7.58 ± 0.41*	6.63 ± 0.15*[b]	9.14 ± 0.62*	5.26 ± 0.62[a,b]	5.49 ± 0.61	5.60 ± 0.58[a,b]	6.49 ± 1.01	6.22 ± 0.68[a,b]	6.25 ± 0.70	21.38 ± 2.82	20.04 ± 2.94	23.84 ± 0.75	18.45 ± 2.48
19:0	0.09 ± < 0.01*	0.23 ± 0.04*	0.10 ± < 0.01*	0.19 ± 0.02*	0.10 ± 0.01	0.12 ± 0.02	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.19 ± 0.04*	0.35 ± 0.07*	0.16 ± 0.02	0.15 ± 0.02
20:0	0.18 ± 0.01*	0.41 ± 0.04*	0.19 ± 0.01*	0.38 ± 0.03*	0.21 ± 0.04	0.26 ± 0.06	0.17 ± 0.04	0.15 ± 0.03	0.17 ± 0.03	0.16 ± 0.02	0.20 ± 0.04*	0.49 ± 0.07*	0.07 ± < 0.01	0.08 ± 0.01
22:0	0.04 ± < 0.01*[a]	0.21 ± 0.05*	0.05 ± 0.01*[a]	0.22 ± 0.04*	0.25 ± 0.06[b]	0.24 ± 0.04	0.16 ± 0.04[b]	0.15 ± 0.03	0.17 ± 0.03[b]	0.17 ± 0.02	0.37 ± 0.06*	0.71 ± 0.12*	0.10 ± 0.01	0.08 ± 0.01
24:0	0.03 ± < 0.01*[a]	0.37 ± 0.12*	0.02 ± < 0.01*[a]	0.19 ± 0.05*	0.47 ± 0.10[b]	0.48 ± 0.08	0.28 ± 0.09[b]	0.27 ± 0.05	0.31 ± 0.06[b]	0.33 ± 0.04	0.10 ± 0.02*	0.60 ± 0.19*	0.08 ± 0.01	0.10 ± 0.01
Σ:0	36.68 ± 0.93*[c]	33.71 ± 0.50*	37.23 ± 0.62*[c]	34.73 ± 0.58*	25.16 ± 1.60 [a]	27.43 ± 2.01	28.78 ± 1.58[b]	28.92 ± 1.10	28.18 ± 1.20[a,b]	28.28 ± 0.83	51.71 ± 6.92	60.64 ± 8.81	50.69 ± 0.88*	45.83 ± 1.57*
14:1n-9	0.025 ± 0.002*[a]	0.111 ± 0.038*	0.022 ± 0.002*[a]	0.066 ± 0.014*	0.050 ± 0.007[b]	0.049 ± 0.010	0.040 ± 0.012[a,b]	0.030 ± 0.004	0.026 ± 0.006[a]	0.027 ± 0.004	0.080 ± 0.044	0.117 ± 0.031	0.012 ± 0.003*	0.045 ± 0.015*
14:1n-7	0.064 ± 0.002*[a]	0.169 ± 0.036*	0.059 ± 0.002*[a]	0.112 ± 0.010*	0.107 ± 0.008 [c]	0.106 ± 0.007	0.085 ± 0.010[b]	0.091 ± 0.008	0.066 ± 0.007[a,b]	0.073 ± 0.005	0.026 ± 0.004*	0.091 ± 0.023*	0.051 ± 0.013	0.073 ± 0.018
14:1n-5	0.211 ± 0.015*[b]	0.344 ± 0.039*	0.139 ± 0.007*[a]	0.229 ± 0.020*	1.634 ± 0.190[d]	1.734 ± 0.205	0.693 ± 0.075*[c]	1.046 ± 0.137*	0.642 ± 0.065*[c]	0.845 ± 0.043*	0.049 ± 0.012*	0.102 ± 0.014*	0.016 ± 0.004	0.071 ± 0.027
15:1n-8	0.027 ± 0.001*[a]	0.135 ± 0.042*	0.026 ± 0.001*[a]	0.077 ± 0.013*	0.106 ± 0.018[b]	0.196 ± 0.058	0.083 ± 0.018[b]	0.086 ± 0.010	0.075 ± 0.014[b]	0.113 ± 0.046	0.263 ± 0.117	0.319 ± 0.066	0.090 ± 0.024	0.053 ± 0.015
15:1n-6	0.013 ± 0.001*[b]	0.103 ± 0.040*	0.009 ± 0.001*[a]	0.051 ± 0.013*	0.060 ± 0.004[d]	0.067 ± 0.006	0.037 ± 0.006[c]	0.040 ± 0.004	0.028 ± 0.003[c]	0.032 ± 0.003	0.021 ± 0.007*	0.110 ± 0.022*	0.018 ± 0.004	0.017 ± 0.005
16:1n-9	0.520 ± 0.011*[b]	0.589 ± 0.016*	0.511 ± 0.009*[b]	0.558 ± 0.019*	0.502 ± 0.035[b]	0.443 ± 0.012	0.518 ± 0.027[b]	0.481 ± 0.017	0.442 ± 0.017[a]	0.429 ± 0.015	0.166 ± 0.025	0.164 ± 0.013	0.238 ± 0.047	0.334 ± 0.081
16:1n-7	4.468 ± 0.235*[b]	2.476 ± 0.197*	3.600 ± 0.109*[a]	1.989 ± 0.151*	11.130 ± 0.783[d]	11.000 ± 1.134	7.900 ± 0.838[c]	7.486 ± 1.086	7.960 ± 0.553[c]	7.734 ± 0.794	0.573 ± 0.089	0.428 ± 0.091	0.973 ± 0.171	1.280 ± 0.344
16:1n-5	0.087 ± 0.003*[a]	0.180 ± 0.043*	0.082 ± 0.003*[a]	0.124 ± 0.018*	0.184 ± 0.014[c]	0.220 ± 0.020	0.154 ± 0.015[b,c]	0.151 ± 0.013	0.142 ± 0.009[b]	0.138 ± 0.008	0.089 ± 0.013	0.108 ± 0.015	0.318 ± 0.077	0.304 ± 0.114
17:1-br	0.164 ± 0.008*[b]	0.355 ± 0.040*	0.165 ± 0.008*[b]	0.351 ± 0.033*	0.136 ± 0.013[a,b]	0.137 ± 0.007	0.135 ± 0.011[a,b]	0.154 ± 0.017	0.112 ± 0.009[a]	0.124 ± 0.011	0.203 ± 0.040	0.284 ± 0.025	0.408 ± 0.095	0.308 ± 0.070
17:1n-8	0.192 ± 0.010[a]	0.204 ± 0.031	0.165 ± 0.006[a]	0.149 ± 0.009	0.384 ± 0.018[c]	0.362 ± 0.025	0.346 ± 0.025[b,c]	0.282 ± 0.024	0.342 ± 0.014[b]	0.315 ± 0.018	0.104 ± 0.023	0.179 ± 0.038	0.099 ± 0.016	0.071 ± 0.016
18:1n-9	28.85 ± 0.90	26.55 ± 1.37	28.61 ± 0.74*	26.03 ± 0.98*	29.23 ± 1.64	27.10 ± 1.77	30.09 ± 1.51	28.25 ± 1.27	30.44 ± 1.59	29.29 ± 1.25	4.13 ± 0.88	3.73 ± 1.24	5.72 ± 0.89	8.24 ± 1.72
18:1n-7	2.528 ± 0.071[a]	2.399 ± 0.032	2.536 ± 0.055[a]	2.445 ± 0.043	3.412 ± 0.164[b]	3.154 ± 0.086	3.345 ± 0.120[b]	3.118 ± 0.071	3.520 ± 0.145[b]	3.236 ± 0.091	1.063 ± 0.225	1.190 ± 0.363	1.966 ± 0.107	2.220 ± 0.075
18:1n-5	0.132 ± 0.004	0.196 ± 0.031	0.131 ± 0.004	0.132 ± 0.006	0.154 ± 0.016	0.149 ± 0.008	0.154 ± 0.020	0.119 ± 0.007	0.139 ± 0.012	0.133 ± 0.010	0.061 ± 0.010*	0.151 ± 0.047*	0.088 ± 0.018	0.079 ± 0.011
19:1n-10	0.136 ± 0.003*[a]	0.226 ± 0.029*	0.129 ± 0.003*[a]	0.168 ± 0.007*	0.154 ± 0.006[b]	0.155 ± 0.009	0.132 ± 0.004[a]	0.134 ± 0.005	0.130 ± 0.006[a]	0.132 ± 0.007	0.045 ± 0.008*	0.113 ± 0.024*	0.075 ± 0.013	0.069 ± 0.013
19:1n-8	0.066 ± 0.002*	0.179 ± 0.041*	0.064 ± 0.001*	0.105 ± 0.009*	0.084 ± 0.014	0.095 ± 0.012	0.081 ± 0.008	0.070 ± 0.002	0.078 ± 0.012	0.069 ± 0.003	0.036 ± 0.006*	0.138 ± 0.022*	0.025 ± 0.004	0.037 ± 0.005
20:1n-11	1.126 ± 0.047*[b]	2.023 ± 0.138*	1.240 ± 0.047*[b]	2.223 ± 0.141*	0.640 ± 0.064[a]	0.692 ± 0.080	0.767 ± 0.096[a]	0.974 ± 0.090	0.925 ± 0.232[a,b]	0.959 ± 0.099	0.074 ± 0.004*	0.170 ± 0.033*	0.099 ± 0.014*	0.203 ± 0.034*
20:1n-9	2.493 ± 0.095*[b]	3.618 ± 0.212*	2.739 ± 0.135*[b]	3.580 ± 0.167*	0.997 ± 0.082[a]	1.047 ± 0.139	1.173 ± 0.113[a]	1.358 ± 0.093	1.264 ± 0.231[a]	1.328 ± 0.125	0.186 ± 0.037	0.265 ± 0.081	0.355 ± 0.033	0.475 ± 0.056
20:1n-7	0.141 ± 0.005*[b]	0.250 ± 0.016*	0.149 ± 0.004*[b]	0.209 ± 0.011*	0.082 ± 0.009[a]	0.096 ± 0.011	0.080 ± 0.012[a]	0.091 ± 0.008	0.079 ± 0.007[a]	0.079 ± 0.005	0.061 ± 0.009	0.074 ± 0.011	0.058 ± 0.009	0.050 ± 0.006
20:1n-5	0.026 ± 0.002*	0.091 ± 0.021*	0.026 ± 0.002*	0.047 ± 0.005*	0.025 ± 0.007	0.023 ± 0.004	0.018 ± 0.002	0.018 ± 0.002	0.015 ± 0.004	0.015 ± 0.002	0.040 ± 0.012	0.070 ± 0.014	0.004 ± 0.001	0.005 ± 0.001
22:1n-11	1.118 ± 0.065*[b]	1.766 ± 0.114*	1.340 ± 0.071*[c]	1.821 ± 0.113*	0.307 ± 0.042[a]	0.322 ± 0.037	0.290 ± 0.041[a]	0.309 ± 0.049	0.267 ± 0.032[a]	0.282 ± 0.029	0.197 ± 0.015	0.176 ± 0.039	0.172 ± 0.014	0.152 ± 0.019
22:1n-9	0.167 ± 0.008*[b]	0.389 ± 0.047*	0.179 ± 0.008*[b]	0.321 ± 0.024*	0.174 ± 0.026[b]	0.216 ± 0.040	0.113 ± 0.021[a]	0.122 ± 0.023	0.103 ± 0.018[a]	0.121 ± 0.015	0.065 ± 0.017	0.101 ± 0.013	0.048 ± 0.009	0.045 ± 0.007
22:1n-7	0.030 ± 0.002*[a]	0.120 ± 0.030*	0.027 ± 0.002*[a]	0.065 ± 0.010*	0.067 ± 0.013[c]	0.107 ± 0.022	0.051 ± 0.017[b,c]	0.041 ± 0.005	0.036 ± 0.009[a,b]	0.031 ± 0.003	0.068 ± 0.025	0.128 ± 0.022	0.012 ± 0.003	0.012 ± 0.004
24:1n-9	0.084 ± 0.006*[a]	0.543 ± 0.138*	0.088 ± 0.011*[a]	0.480 ± 0.096*	0.711 ± 0.127[b]	0.689 ± 0.110	0.507 ± 0.141[b]	0.460 ± 0.091	0.480 ± 0.089[b]	0.450 ± 0.052	0.349 ± 0.110	0.924 ± 0.249	0.128 ± 0.021	0.192 ± 0.026
Σ:1	42.67 ± 1.24[a,b]	43.01 ± 1.16	42.04 ± 1.02[a]	41.33 ± 0.99	50.33 ± 1.86[c]	48.16 ± 2.75	46.80 ± 2.18[a,b,c]	44.90 ± 2.13	47.31 ± 2.16[b,c]	45.95 ± 1.99	7.95 ± 1.16	9.13 ± 1.76	10.97 ± 1.48	14.33 ± 2.28
Δ9-DI	1.12 ± 0.07[a]	1.10 ± 0.04	1.05 ± 0.04[a]	1.02 ± 0.04	2.16 ± 0.23[b]	1.93 ± 0.22	1.70 ± 0.19[b]	1.59 ± 0.15	1.72 ± 0.14[b]	1.65 ± 0.13	0.16 ± 0.04	0.16 ± 0.05	0.18 ± 0.03	0.30 ± 0.07
16:2n-4	0.155 ± 0.007	0.146 ± 0.024	0.143 ± 0.007*	0.104 ± 0.008*	0.152 ± 0.005	0.141 ± 0.008	0.133 ± 0.012	0.120 ± 0.014	0.122 ± 0.010	0.111 ± 0.009	0.061 ± 0.006*	0.196 ± 0.043*	0.046 ± 0.010	0.064 ± 0.018
18:2n-7	0.100 ± 0.008[a]	0.168 ± 0.033	0.089 ± 0.004[a]	0.099 ± 0.008	0.143 ± 0.013[b]	0.151 ± 0.006	0.131 ± 0.013[b]	0.110 ± 0.011	0.128 ± 0.016[b]	0.123 ± 0.010	0.051 ± 0.008*	0.085 ± 0.013*	0.094 ± 0.012	0.075 ± 0.022
18:2n-6	13.52 ± 0.17	13.23 ± 0.30	13.67 ± 0.18	13.83 ± 0.23	12.64 ± 0.64	11.80 ± 0.25	13.79 ± 0.44	13.69 ± 0.26	13.42 ± 0.19	13.77 ± 0.26	16.03 ± 3.78	6.99 ± 2.38	9.93 ± 0.41	11.53 ± 0.81
18:2n-4	0.111 ± 0.003[b]	0.159 ± 0.026	0.099 ± 0.004*[a]	0.119 ± 0.008*	0.141 ± 0.023[a,b]	0.125 ± 0.008	0.113 ± 0.005[a,b]	0.109 ± 0.005	0.109 ± 0.003[a,b]	0.108 ± 0.005	0.074 ± 0.013*	0.184 ± 0.060*	0.080 ± 0.006	0.070 ± 0.008
*c,t*18:2	0.060 ± 0.005[b]	0.149 ± 0.049	0.047 ± 0.002*[a]	0.071 ± 0.008*	0.143 ± 0.014[c]	0.234 ± 0.093	0.110 ± 0.019[c]	0.095 ± 0.010	0.110 ± 0.010[c]	0.103 ± 0.007	0.040 ± 0.008*	0.182 ± 0.040*	0.014 ± 0.004	0.023 ± 0.004
20:2n-6	0.168 ± 0.003*[a]	0.254 ± 0.015*	0.171 ± 0.004*[a]	0.248 ± 0.012*	0.214 ± 0.008[b]	0.337 ± 0.140	0.211 ± 0.016[b]	0.223 ± 0.016	0.225 ± 0.012[b]	0.218 ± 0.010	0.214 ± 0.056	0.286 ± 0.058	0.151 ± 0.005	0.174 ± 0.016
Σ:2	14.12 ± 0.16	14.11 ± 0.21	14.22 ± 0.18	14.47 ± 0.22	13.43 ± 0.61	12.79 ± 0.25	14.48 ± 0.43	14.35 ± 0.26	14.12 ± 0.19	14.43 ± 0.26	16.47 ± 3.73*	7.93 ± 2.28*	10.31 ± 0.39	11.94 ± 0.81
18:3n-6	0.137 ± 0.005[c]	0.155 ± 0.028	0.125 ± 0.004*[c]	0.102 ± 0.005*	0.103 ± 0.006[b]	0.102 ± 0.008	0.104 ± 0.005[b]	0.085 ± 0.007	0.081 ± 0.004[a]	0.077 ± 0.003	0.282 ± 0.080	0.227 ± 0.032	0.227 ± 0.035	0.219 ± 0.050
18:3n-3	0.911 ± 0.025*[b]	0.587 ± 0.026*	0.894 ± 0.027*[b]	0.544 ± 0.031*	0.751 ± 0.045[a]	0.657 ± 0.040	0.761 ± 0.045[a]	0.654 ± 0.068	0.734 ± 0.027[a]	0.667 ± 0.049	0.312 ± 0.083	0.217 ± 0.035	0.286 ± 0.011	0.377 ± 0.099
20:3n-6	0.175 ± 0.006*[a]	0.328 ± 0.043*	0.183 ± 0.010*[a]	0.356 ± 0.038*	0.412 ± 0.039[b]	0.453 ± 0.052	0.360 ± 0.032[b]	0.392 ± 0.047	0.380 ± 0.054[b]	0.414 ± 0.052	0.553 ± 0.142	0.480 ± 0.161	0.901 ± 0.061*	0.502 ± 0.052*
20:3n-3	0.040 ± 0.002*[a]	0.133 ± 0.043*	0.038 ± 0.002*[a]	0.064 ± 0.008*	0.125 ± 0.015[c]	0.157 ± 0.031	0.071 ± 0.007[b]	0.075 ± 0.008	0.063 ± 0.009[b]	0.052 ± 0.003	0.055 ± 0.012*	0.116 ± 0.014*	0.038 ± 0.005	0.041 ± 0.003

(continued)

TABLE 1 (continued)

FA	Total sc fat		Total iab fat		Tail tip		Front paws		Rear paws		Plasma		Liver	
	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
Σ:3	1.263 ± 0.032	1.203 ± 0.118	1.240 ± 0.037*	1.066 ± 0.042*	1.391 ± 0.043	1.369 ± 0.065	1.296 ± 0.027	1.205 ± 0.039	1.258 ± 0.053	1.210 ± 0.042	1.202 ± 0.283	1.041 ± 0.196	1.453 ± 0.084*	1.139 ± 0.110*
18:4n-3	0.287 ± 0.015^{b}	0.246 ± 0.035	0.278 ± 0.014^{ab}	0.187 ± 0.015*	0.216 ± 0.018^{a}	0.193 ± 0.012	0.186 ± 0.017^{a}	0.161 ± 0.018	0.172 ± 0.018^{a}	0.145 ± 0.010	0.126 ± 0.013*	0.199 ± 0.027*	0.050 ± 0.009	0.113 ± 0.038
18:4n-1	0.049 ± 0.003^{a}	0.081 ± 0.028	0.041 ± 0.002^{a}	0.042 ± 0.008	0.090 ± 0.004^{c}	0.087 ± 0.008	0.068 ± 0.002^{b}	0.057 ± 0.007	0.064 ± 0.007^{b}	0.056 ± 0.006	0.046 ± 0.014*	0.122 ± 0.030*	0.031 ± 0.006	0.036 ± 0.009
20:4n-6	0.445 ± 0.022*^{a}	1.415 ± 0.349*	0.516 ± 0.101*^{a}	2.209 ± 0.438*	2.476 ± 0.261^{b}	2.907 ± 0.457	2.184 ± 0.258^{b}	2.827 ± 0.470	2.436 ± 0.446^{b}	2.803 ± 0.463	6.859 ± 1.837	6.038 ± 2.163	7.768 ± 0.676	6.692 ± 0.847
20:4n-3	0.143 ± 0.006^{a,b}	0.245 ± 0.064	0.139 ± 0.006^{a}	0.155 ± 0.011	0.195 ± 0.006^{c}	0.200 ± 0.016	0.167 ± 0.011^{b}	0.145 ± 0.003	0.152 ± 0.007^{a,b}	0.142 ± 0.007	0.156 ± 0.023	0.282 ± 0.062	0.112 ± 0.009	0.121 ± 0.019
22:4n-6	0.172 ± 0.009*^{a}	0.489 ± 0.073*	0.189 ± 0.017*^{a}	0.513 ± 0.060*	0.331 ± 0.035^{b}	0.380 ± 0.063	0.320 ± 0.051^{b}	0.371 ± 0.059	0.293 ± 0.047^{b}	0.352 ± 0.044	0.160 ± 0.030	0.499 ± 0.160	0.246 ± 0.041	0.251 ± 0.026
22:4n-3	0.033 ± 0.002*^{a}	0.155 ± 0.045*	0.028 ± 0.002*^{a}	0.072 ± 0.014*	0.101 ± 0.023^{b}	0.103 ± 0.032	0.062 ± 0.018^{a,b}	0.055 ± 0.008	0.036 ± 0.007^{a,b}	0.041 ± 0.008	2.001 ± 0.941	0.580 ± 0.368	0.047 ± 0.012	0.045 ± 0.010
Σ:4	1.128 ± 0.052*^{a}	2.631 ± 0.564*	1.190 ± 0.125*^{a}	3.178 ± 0.517*	3.408 ± 0.337^{b}	3.868 ± 0.531	2.987 ± 0.311^{b}	3.616 ± 0.515	3.152 ± 0.498^{b}	3.538 ± 0.511	9.347 ± 0.917	7.720 ± 2.200	8.254 ± 0.671	7.258 ± 0.799
20:5n-3	0.646 ± 0.046^{a}	0.765 ± 0.197	0.629 ± 0.067^{a}	0.807 ± 0.135	1.901 ± 0.152^{b}	1.791 ± 0.147	1.672 ± 0.164^{b}	1.696 ± 0.206	1.801 ± 0.266^{b}	1.723 ± 0.217	5.559 ± 1.699	2.132 ± 0.742	5.424 ± 0.301*	3.578 ± 0.276*
21:5n-3	0.096 ± 0.006^{ab}	0.192 ± 0.038*	0.091 ± 0.006^{b}	0.114 ± 0.010	0.133 ± 0.029^{ab}	0.143 ± 0.031	0.085 ± 0.010^{a,b}	0.081 ± 0.003	0.063 ± 0.011^{a}	0.059 ± 0.004	0.147 ± 0.088	0.123 ± 0.026	0.026 ± 0.003	0.049 ± 0.016
22:5n-6	0.164 ± 0.011*	0.477 ± 0.109*	0.160 ± 0.011*	0.310 ± 0.027*	0.166 ± 0.020	0.183 ± 0.024	0.147 ± 0.016	0.212 ± 0.022	0.142 ± 0.023	0.169 ± 0.016	3.933 ± 1.792	3.092 ± 1.664	0.246 ± 0.030*	0.366 ± 0.036*
22:5n-3	0.695 ± 0.028*^{a}	1.132 ± 0.118*	0.703 ± 0.032*^{a}	1.129 ± 0.077*	0.978 ± 0.054^{b}	1.097 ± 0.086	0.959 ± 0.066^{b}	1.092 ± 0.083	0.963 ± 0.076^{a,b}	1.035 ± 0.082	0.591 ± 0.182	1.125 ± 0.423	1.371 ± 0.069	1.372 ± 0.061
Σ:5	1.601 ± 0.084*^{a}	2.567 ± 0.448*	1.583 ± 0.107*^{a}	2.359 ± 0.233*	3.177 ± 0.247^{b}	3.215 ± 0.258	2.863 ± 0.225^{b}	3.080 ± 0.285	2.969 ± 0.363^{b}	2.985 ± 0.310	10.231 ± 0.536*	6.472 ± 1.350*	7.067 ± 0.377*	5.365 ± 0.295*
22:6n-3	2.541 ± 0.159	2.772 ± 0.183	2.501 ± 0.151	2.868 ± 0.135	3.112 ± 0.280	3.173 ± 0.240	2.792 ± 0.304	3.933 ± 0.345	3.013 ± 0.329	3.600 ± 0.414	3.094 ± 1.059	7.068 ± 2.365	11.262 ± 0.658	14.145 ± 1.167
ΣPUFA	20.65 ± 0.42*^{a}	23.28 ± 1.20*	20.73 ± 0.50*^{a}	23.95 ± 0.90*	24.52 ± 0.51^{b}	24.41 ± 0.89	24.42 ± 0.82^{b}	26.18 ± 1.10	24.51 ± 1.20^{b}	25.76 ± 1.25	40.34 ± 5.83	30.23 ± 7.11	38.35 ± 1.84	39.85 ± 1.45
Σ:n-6	14.79 ± 0.20*^{a}	16.35 ± 0.41*	15.01 ± 0.26*^{a}	17.57 ± 0.56*	16.34 ± 0.47^{b}	16.16 ± 0.52	17.11 ± 0.48^{b}	17.80 ± 0.59	16.98 ± 0.58^{b}	17.80 ± 0.66	28.03 ± 4.01	17.62 ± 4.01	19.46 ± 1.02	19.74 ± 0.72
Σ:n-3	5.39 ± 0.27^{a}	6.23 ± 0.69	5.30 ± 0.28^{a}	5.94 ± 0.36	7.51 ± 0.51^{b}	7.51 ± 0.45	6.75 ± 0.47^{b}	7.89 ± 0.57	7.00 ± 0.68^{b}	7.46 ± 0.64	12.04 ± 2.04	11.84 ± 3.39	18.62 ± 0.98	19.84 ± 1.01
n–3/n-6	0.36 ± 0.02^{a}	0.37 ± 0.03	0.35 ± 0.02^{a}	0.33 ± 0.01	0.46 ± 0.04^{b}	0.46 ± 0.02	0.39 ± 0.02^{a,b}	0.44 ± 0.02	0.41 ± 0.03^{a,b}	0.41 ± 0.02	0.42 ± 0.03	0.65 ± 0.15	0.96 ± 0.04	1.01 ± 0.04
ΣVLCFA	0.111 ± 0.009*^{a}	0.916 ± 0.254*	0.113 ± 0.015*^{a}	0.672 ± 0.141*	1.180 ± 0.231^{b}	1.169 ± 0.190	0.784 ± 0.227^{b}	0.734 ± 0.141	0.795 ± 0.151^{b}	0.775 ± 0.088	0.445 ± 0.124*	1.528 ± 0.170*	0.213 ± 0.025	0.287 ± 0.035
UFA/SFA	1.76 ± 0.08*^{a}	1.98 ± 0.04*	1.71 ± 0.05*^{a}	1.91 ± 0.06*	3.08 ± 0.29^{b}	2.77 ± 0.25	2.54 ± 0.21^{b}	2.49 ± 0.13	2.59 ± 0.16^{b}	2.56 ± 0.11	1.15 ± 0.26	0.93 ± 0.28	0.98 ± 0.03*	1.20 ± 0.09*
DBI	0.51 ± 0.01^{a}	0.52 ± 0.01	0.51 ± 0.01^{a}	0.51 ± 0.01	0.60 ± 0.02^{b}	0.57 ± 0.03	0.56 ± 0.02^{b}	0.55 ± 0.02	0.57 ± 0.02^{b}	0.56 ± 0.02	0.21 ± 0.03	0.18 ± 0.04	0.22 ± 0.01	0.26 ± 0.02
TACL	17.29 ± 0.03*^{a}	17.54 ± 0.04*	17.37 ± 0.02*^{a,b}	17.62 ± 0.04*	17.28 ± 0.07^{a}	17.21 ± 0.06	17.39 ± 0.03^{a,b}	17.39 ± 0.09	17.48 ± 0.04^{b}	17.44 ± 0.06	17.98 ± 0.12	17.74 ± 0.25	18.20 ± 0.07	18.18 ± 0.13

[a]An asterisk (*) indicates a significant difference between the fed and fasted animals (*t*-test, Mann–Whitney U test, $P < 0.05$); dissimilar superscripts indicate that the values of subcutaneous (sc), intra-abdominal (iab), and extremity fats of the fed group differ at $P < 0.05$ (one-way ANOVA). Δ9-DI = Δ9-desaturation index, VLCFA = very long chain FA (chain length ≥ 24 C), UFA = unsaturated FA, SFA = saturated FA, DBI = double bond index, TACL = total average chain length.

RESULTS

The average initial (fed: 884 ± 70 g, fasted: 849 ± 63 g) or final (fasted: 734 ± 56 g) BM of the marten did not differ between the experimental groups. The fasted marten lost approximately 113 g or 13.4% of BM during the 2-d fasting period. The absolute or relative masses of the different fat depots did not differ between the experimental groups (sc interscapular: fed 0.27 ± 0.05 vs. fasted 0.15 ± 0.04 g; sc rump: 0.16 ± 0.05 vs. 0.15 ± 0.05 g; sc ventral: 1.29 ± 0.57 vs. 0.61 ± 0.16 g; iab om: 4.94 ± 1.66 vs. 1.93 ± 0.61 g; iab mes: 2.44 ± 0.78 vs. 1.19 ± 0.24 g; iab dia: 0.68 ± 0.27 vs. 0.33 ± 0.11 g; iab rp: 5.58 ± 2.18 vs. 3.29 ± 1.79 g; intermuscular: 8.03 ± 0.85 vs. 7.52 ± 0.57 g; total fat percentage: 2.74 ± 0.56 vs. 2.06 ± 0.24%, $P > 0.05$). The total fat mass (23.4 ± 4.4 vs. 15.2 ± 2.2 g) was lower in the fasted animals ($P < 0.05$). The calculated BMI correlated strongly with the total fat mass ($rs = 0.604$, $P < 0.05$) and the fat percentage ($rs = 0.804$, $P < 0.01$). The initial BMI did not differ between the experimental groups, but the BMI was significantly lower in the fasted animals at the end of the study (12.9 ± 0.7 vs. 10.6 ± 0.2 kg $(m^3)^{-1}$; $P < 0.05$). Many of the marten in the fasted group were physically active and exhibited stereotypical behavior and signs of stress, such as hair biting.

The most abundant FA (≥1 mol%) in the sc and iab fat depots of the marten were 12:0, 14:0, 16:0, 18:0, 16:1n-7, 18:1n-9, 18:1n-7, 20:1n-11, 20:1n-9, 22:1n-11, 18:2n-6, and 22:6n-3 (Table 1). In the extremities the pattern was mostly the same, but the proportions of 12:0, 20:1n-11, and 20:1n-9 were clearly lower, whereas the proportions of 14:1n-5, 20:4n-6, and 20:5n-3 were higher than in the trunk fat. Generally, the extremities had higher proportions of unsaturated FA (UFA) than the iab or sc depots. In fact, the DBI, Δ9-DI, total PUFA, and the sums of 4n- and 5n-PUFA were all higher in the extremities. In the plasma and livers the proportions of SFA and particular PUFA were higher than in any of the fat depots; in particular, the proportions of 16:0 and 18:0 were two to three times higher than in the WAT, and also the proportions of 20:4n-6 and 20:5n-3 were elevated. On the other hand, the proportions of certain monoenes, especially 18:1n-9 and 18:1n-7, were fairly low in the plasma and livers.

Food deprivation caused numerous differences in the proportions of FA in the fat depots (Tables 1–3). The sum of SFA decreased in the sc scapular and iab rp depots and in the pooled trunk fats, mostly owing to significant decreases in the proportion of 16:0, whereas the proportions of 18:0, 19:0, 20:0, 22:0, and 24:0 mostly increased in the fasted animals. Also the proportions of most MUFA were higher in the fasted group in most fat depots with the exception of 16:1n-7, the proportions of which decreased in all sc and iab tissues. In the PUFA the proportions of 20:2n-6, 20:3n-6, 22:4n-6, 22:4n-3, 22:5n-6, and 22:5n-3 increased, whereas the proportions of 18:3n-3 and 18:4n-3 were lower in the fasted marten. In the pooled trunk fats, the proportions of 4n- and 5n-PUFA increased due to fasting. In a similar manner, the proportions of n-6 PUFA and VLCFA as well as the TACL were higher in the fasted group in the pooled sc and iab fats. In the extremities, the effects of fasting were minor. In plasma, the proportions of several SFA,

TABLE 2
Effects of Fasting on the Composition of Saturated and Monounsaturated FA (mol%) in Adipose Tissues of the American Marten[a] (mean ± SE)

	Scapular sc fat		Rump sc fat		Ventral sc fat		Mesenteric fat		Omental fat		Retroperitoneal fat		Diaphragmatic fat	
FA	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
12:0	1.50 ± 0.15	1.22 ± 0.15	1.26 ± 0.22	1.28 ± 0.13	1.39 ± 0.17	1.31 ± 0.18	1.38 ± 0.18	1.17 ± 0.15	1.35 ± 0.17	1.29 ± 0.12	1.19 ± 0.16	1.05 ± 0.13	1.15 ± 0.29	1.21 ± 0.16
14:0	8.18 ± 0.67	6.56 ± 1.06	7.28 ± 0.96	6.90 ± 0.90	7.95 ± 0.77	6.84 ± 1.05	7.50 ± 0.84	5.95 ± 0.83	7.01 ± 0.67	6.42 ± 0.79	7.22 ± 0.74	5.69 ± 0.95	6.80 ± 1.22	6.39 ± 0.93
15:0	0.31 ± 0.01	0.27 ± 0.02	0.30 ± 0.02	0.27 ± 0.02	0.30 ± 0.01	0.34 ± 0.09	0.31 ± 0.01	0.28 ± 0.02	0.31 ± 0.01*	0.26 ± 0.02*	0.29 ± 0.01*	0.23 ± 0.02*	0.29 ± 0.02*	0.24 ± 0.01*
16:0	20.48 ± 0.47*	15.12 ± 1.15*	19.50 ± 0.56*	14.66 ± 1.00*	20.02 ± 0.53*	15.31 ± 0.95*	20.87 ± 0.32*	17.20 ± 0.84*	20.59 ± 0.30*	17.16 ± 1.12*	20.17 ± 0.31*	14.04 ± 0.87*	20.19 ± 0.28*	14.74 ± 0.99*
17:0	0.39 ± 0.02	0.44 ± 0.06	0.38 ± 0.02	0.42 ± 0.07	0.39 ± 0.02	0.43 ± 0.07	0.42 ± 0.02	0.46 ± 0.03	0.43 ± 0.02	0.42 ± 0.03	0.40 ± 0.01	0.44 ± 0.05	0.40 ± 0.02	0.41 ± 0.04
18:0	5.53 ± 0.27*	7.61 ± 0.61*	5.72 ± 0.30	7.55 ± 0.97	5.92 ± 0.26*	7.57 ± 0.61*	6.63 ± 0.48*	9.96 ± 1.09*	6.38 ± 0.10	8.28 ± 1.15	6.84 ± 0.22	8.81 ± 1.52	6.69 ± 0.13	9.53 ± 1.35
19:0	0.09 ± 0.01*	0.28 ± 0.09*	0.10 ± 0.01	0.20 ± 0.05	0.10 ± 0.01*	0.21 ± 0.06*	0.10 ± 0.01*	0.21 ± 0.04*	0.09 ± 0.01*	0.15 ± 0.03*	0.11 ± 0.01*	0.21 ± 0.03*	0.09 ± 0.01*	0.19 ± 0.03*
20:0	0.15 ± 0.01*	0.43 ± 0.10*	0.19 ± 0.02*	0.39 ± 0.06*	0.20 ± 0.01*	0.42 ± 0.06*	0.19 ± 0.02*	0.38 ± 0.05*	0.17 ± 0.01*	0.29 ± 0.06*	0.23 ± 0.01*	0.46 ± 0.05*	0.20 ± 0.02*	0.38 ± 0.05*
22:0	0.04 ± 0.01	0.23 ± 0.10	0.04 ± 0.01*	0.17 ± 0.05*	0.04 ± < 0.01*	0.25 ± 0.11*	0.07 ± 0.03*	0.22 ± 0.06*	0.04 ± < 0.01	0.15 ± 0.08	0.05 ± < 0.01*	0.28 ± 0.11*	0.04 ± < 0.01*	0.22 ± 0.09*
24:0	0.03 ± 0.01	0.37 ± 0.22	0.03 ± < 0.01	0.22 ± 0.11	0.02 ± < 0.01*	0.53 ± 0.28*	0.04 ± 0.02*	0.24 ± 0.09*	0.02 ± < 0.01	0.12 ± 0.08	0.02 ± < 0.01*	0.23 ± 0.12*	0.02 ± < 0.01*	0.17 ± 0.09*
Σ:0	37.34 ± 1.35*	33.78 ± 0.93*	35.39 ± 2.05	33.11 ± 0.80	36.95 ± 1.70	34.23 ± 0.91	38.09 ± 1.24	37.05 ± 0.85	36.95 ± 1.08	35.31 ± 0.72	37.09 ± 1.29*	32.22 ± 1.55*	36.47 ± 1.90	34.33 ± 0.76
14:1n-5	0.225 ± 0.032	0.320 ± 0.079	0.213 ± 0.031	0.373 ± 0.061	0.195 ± 0.020	0.339 ± 0.071	0.155 ± 0.017*	0.264 ± 0.041*	0.124 ± 0.006	0.206 ± 0.040	0.139 ± 0.013	0.230 ± 0.046	0.139 ± 0.016	0.218 ± 0.042
16:1n-9	0.548 ± 0.019	0.615 ± 0.033	0.520 ± 0.017	0.572 ± 0.024	0.491 ± 0.017*	0.581 ± 0.023*	0.494 ± 0.021	0.530 ± 0.030	0.528 ± 0.019	0.550 ± 0.025	0.511 ± 0.011	0.590 ± 0.043	0.511 ± 0.029	0.562 ± 0.049
16:1n-7	4.539 ± 0.410*	2.277 ± 0.320*	4.620 ± 0.498*	2.782 ± 0.428*	4.287 ± 0.393*	2.370 ± 0.278*	3.744 ± 0.293*	2.093 ± 0.300*	3.651 ± 0.138*	2.510 ± 0.316*	3.432 ± 0.190*	1.555 ± 0.220*	3.554 ± 0.269*	1.796 ± 0.300*
17:1n-8	0.201 ± 0.015	0.192 ± 0.050	0.191 ± 0.021	0.205 ± 0.040	0.184 ± 0.017	0.216 ± 0.073	0.170 ± 0.013	0.161 ± 0.016	0.172 ± 0.008	0.156 ± 0.011	0.154 ± 0.012	0.144 ± 0.024	0.163 ± 0.015	0.135 ± 0.023
18:1n-9	28.68 ± 1.27	25.96 ± 2.98	29.64 ± 2.20	26.81 ± 2.18	28.47 ± 1.61	26.87 ± 2.21	27.60 ± 1.53	23.96 ± 1.85	29.31 ± 1.30	27.70 ± 1.88	28.48 ± 1.40	26.08 ± 2.16	29.37 ± 2.32	26.37 ± 2.10
18:1n-7	2.548 ± 0.112	2.432 ± 0.043	2.523 ± 0.150	2.333 ± 0.070	2.513 ± 0.134	2.431 ± 0.049	2.517 ± 0.120	2.534 ± 0.057	2.587 ± 0.122	2.465 ± 0.100	2.506 ± 0.096	2.419 ± 0.083	2.530 ± 0.118	2.363 ± 0.099
20:1n-11	1.064 ± 0.069*	1.807 ± 0.268*	1.170 ± 0.121*	2.261 ± 0.233*	1.155 ± 0.074*	2.000 ± 0.212*	1.178 ± 0.108*	1.721 ± 0.144*	1.203 ± 0.098*	1.831 ± 0.203*	1.267 ± 0.056*	2.970 ± 0.304*	1.369 ± 0.134*	2.369 ± 0.236*
20:1n-9	2.253 ± 0.161*	3.226 ± 0.444*	2.595 ± 0.172*	4.039 ± 0.249*	2.660 ± 0.137*	3.588 ± 0.370*	2.435 ± 0.236	2.973 ± 0.258	2.647 ± 0.262	3.218 ± 0.253	2.930 ± 0.232*	4.364 ± 0.344*	3.095 ± 0.413	3.767 ± 0.285
22:1n-11	0.872 ± 0.055*	1.375 ± 0.094*	1.231 ± 0.123*	2.114 ± 0.219*	1.282 ± 0.088*	1.809 ± 0.176*	1.187 ± 0.100	1.518 ± 0.143	1.268 ± 0.183	1.576 ± 0.274	1.452 ± 0.112*	2.190 ± 0.228*	1.537 ± 0.135	2.001 ± 0.185
22:1n-9	0.143 ± 0.008*	0.358 ± 0.073*	0.180 ± 0.021*	0.459 ± 0.121*	0.182 ± 0.012*	0.350 ± 0.030*	0.161 ± 0.012*	0.278 ± 0.026*	0.167 ± 0.016	0.271 ± 0.065	0.202 ± 0.014*	0.375 ± 0.051*	0.191 ± 0.012*	0.360 ± 0.040*
22:1n-7	0.030 ± 0.003*	0.155 ± 0.074*	0.031 ± 0.006	0.086 ± 0.024	0.029 ± 0.003	0.119 ± 0.051	0.030 ± 0.004*	0.062 ± 0.012*	0.023 ± 0.002	0.054 ± 0.023	0.029 ± 0.004*	0.079 ± 0.028*	0.028 ± 0.003*	0.063 ± 0.014*
Σ:1	42.06 ± 1.83	41.62 ± 2.51	43.88 ± 2.90	44.28 ± 1.56	42.41 ± 2.24	43.14 ± 2.00	40.66 ± 2.12	38.18 ± 2.08	42.58 ± 1.82	42.09 ± 1.41	42.07 ± 1.93	43.13 ± 2.12	43.41 ± 3.07	41.90 ± 2.13
Δ9-DI	1.09 ± 0.10	1.07 ± 0.09	1.10 ± 0.14	1.13 ± 0.08	1.10 ± 0.14	1.10 ± 0.07	1.00 ± 0.09	0.89 ± 0.07	1.08 ± 0.08	1.05 ± 0.07	1.04 ± 0.09	1.10 ± 0.08	1.09 ± 0.14	1.02 ± 0.08

[a]An asterisk (*) indicates a significant difference between the fed and the fasted animals (*t*-test, Mann–Whitney U test, $P < 0.05$). The sums include also minor FA not presented in the table. For abbreviations see Table 1.

TABLE 3
Effects of Fasting on the Composition of PUFA (mol%) in Adipose Tissues of the American Marten[a] (mean ± SE)

	Scapular sc fat		Rump sc fat		Ventral sc fat		Mesenteric fat		Omental fat		Retroperitoneal fat		Diaphragmatic fat	
FA	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
18:2n-6	13.28 ± 0.35	13.25 ± 0.63	13.69 ± 0.31	13.52 ± 0.39	13.65 ± 0.21	12.93 ± 0.59	13.36 ± 0.31	12.47 ± 0.41	13.36 ± 0.25	13.65 ± 0.31	14.29 ± 0.37	14.81 ± 0.35	13.66 ± 0.42	14.40 ± 0.28
Σ:2	13.88 ± 0.34	14.27 ± 0.38	14.30 ± 0.29	14.28 ± 0.31	14.23 ± 0.20	13.78 ± 0.42	13.92 ± 0.31	13.21 ± 0.35	13.93 ± 0.25	14.26 ± 0.28	14.82 ± 0.38	15.38 ± 0.37	14.19 ± 0.43	15.05 ± 0.29
18:3n-6	0.138 ± 0.007	0.189 ± 0.058	0.138 ± 0.013	0.117 ± 0.019	0.135 ± 0.008	0.159 ± 0.058	0.124 ± 0.005	0.120 ± 0.014	0.123 ± 0.007*	0.098 ± 0.006*	0.129 ± 0.007*	0.090 ± 0.011*	0.125 ± 0.015	0.100 ± 0.005
18:3n-3	0.907 ± 0.041*	0.622 ± 0.054*	0.923 ± 0.058*	0.572 ± 0.039*	0.906 ± 0.042*	0.567 ± 0.046*	0.912 ± 0.049*	0.553 ± 0.065*	0.904 ± 0.052*	0.670 ± 0.069*	0.890 ± 0.054*	0.419 ± 0.040*	0.853 ± 0.095*	0.533 ± 0.048*
20:3n-6	0.170 ± 0.010*	0.351 ± 0.085*	0.183 ± 0.013	0.332 ± 0.080	0.176 ± 0.008	0.302 ± 0.067	0.200 ± 0.035*	0.444 ± 0.084*	0.164 ± 0.008	0.272 ± 0.061	0.186 ± 0.011*	0.389 ± 0.088*	0.179 ± 0.006*	0.319 ± 0.066*
20:3n-3	0.045 ± 0.004	0.179 ± 0.093	0.038 ± 0.003	0.072 ± 0.024	0.038 ± 0.002	0.149 ± 0.091	0.040 ± 0.004	0.073 ± 0.014	0.040 ± 0.003	0.061 ± 0.017	0.036 ± 0.003	0.069 ± 0.018	0.037 ± 0.006	0.055 ± 0.014
Σ:3	1.259 ± 0.047	1.341 ± 0.271	1.282 ± 0.080	1.093 ± 0.107	1.253 ± 0.053	1.177 ± 0.220	1.276 ± 0.079	1.191 ± 0.079	1.231 ± 0.063	1.101 ± 0.058	1.239 ± 0.065	0.967 ± 0.104	1.193 ± 0.121	1.006 ± 0.083
18:4n-3	0.294 ± 0.023	0.267 ± 0.071	0.280 ± 0.038	0.220 ± 0.030	0.285 ± 0.024	0.252 ± 0.078	0.288 ± 0.025*	0.195 ± 0.025*	0.307 ± 0.031	0.237 ± 0.029	0.252 ± 0.018*	0.131 ± 0.020*	0.256 ± 0.049	0.185 ± 0.035
20:4n-6	0.473 ± 0.040	1.532 ± 0.713	0.429 ± 0.050	1.373 ± 0.593	0.427 ± 0.033	1.340 ± 0.580	0.793 ± 0.353*	3.070 ± 0.889*	0.414 ± 0.038	1.475 ± 0.737	0.411 ± 0.036	2.486 ± 1.024	0.391 ± 0.062	1.805 ± 0.898
Σ:4	1.176 ± 0.082	2.963 ± 1.226	1.104 ± 0.124	2.381 ± 0.807	1.097 ± 0.083	2.549 ± 0.975	1.523 ± 0.419*	4.265 ± 1.062*	1.099 ± 0.098	2.352 ± 0.912	1.050 ± 0.078	3.387 ± 1.180	1.011 ± 0.157	2.705 ± 1.034
20:5n-3	0.714 ± 0.081	0.935 ± 0.478	0.633 ± 0.107	0.624 ± 0.201	0.586 ± 0.065	0.737 ± 0.326	0.822 ± 0.204	1.050 ± 0.255	0.658 ± 0.070	0.703 ± 0.247	0.491 ± 0.049	0.857 ± 0.341	0.483 ± 0.098	0.617 ± 0.255
22:5n-3	0.700 ± 0.041*	1.244 ± 0.294*	0.694 ± 0.069	1.051 ± 0.133	0.690 ± 0.051*	1.101 ± 0.174*	0.765 ± 0.079*	1.353 ± 0.206*	0.694 ± 0.055	1.001 ± 0.149	0.667 ± 0.045*	1.059 ± 0.130*	0.676 ± 0.094*	1.102 ± 0.117*
Σ:5	1.691 ± 0.130	3.016 ± 1.097	1.576 ± 0.206	2.229 ± 0.460	1.528 ± 0.133	2.455 ± 0.720	1.852 ± 0.303	2.858 ± 0.485	1.599 ± 0.148	2.080 ± 0.465	1.408 ± 0.113	2.341 ± 0.540	1.388 ± 0.233	2.158 ± 0.411
22:6n-3	2.594 ± 0.234	3.013 ± 0.459	2.470 ± 0.384	2.623 ± 0.203	2.539 ± 0.284	2.680 ± 0.257	2.673 ± 0.281	3.245 ± 0.282	2.606 ± 0.311	2.805 ± 0.190	2.318 ± 0.245	2.570 ± 0.362	2.334 ± 0.505	2.854 ± 0.202
Σ PUFA	20.60 ± 0.69	24.60 ± 2.83	20.73 ± 0.98	22.61 ± 1.46	20.64 ± 0.70	22.64 ± 1.89	21.25 ± 1.23	24.77 ± 1.61	20.46 ± 0.83	22.60 ± 1.46	20.84 ± 0.81	24.64 ± 2.43	20.12 ± 1.37	23.77 ± 1.81
Σ VLCFA	0.117 ± 0.021	0.976 ± 0.511	0.108 ± 0.012	0.669 ± 0.307	0.107 ± 0.009	1.103 ± 0.514	0.158 ± 0.050*	0.794 ± 0.251*	0.081 ± 0.007	0.463 ± 0.262	0.107 ± 0.010	0.796 ± 0.343	0.099 ± 0.013	0.635 ± 0.300
Σ n-6 PUFA	14.56 ± 0.40*	16.80 ± 0.87*	14.95 ± 0.42	16.39 ± 0.72	14.89 ± 0.28	15.85 ± 0.54	15.05 ± 0.70	17.41 ± 0.90	14.54 ± 0.33	16.36 ± 0.83	15.55 ± 0.45	18.86 ± 1.52	14.84 ± 0.55	17.65 ± 1.17
Σ n-3 PUFA	5.54 ± 0.41	6.94 ± 1.65	5.31 ± 0.67	5.64 ± 0.69	5.30 ± 0.47	6.11 ± 1.19	5.77 ± 0.61	6.85 ± 0.72	5.48 ± 0.53	5.79 ± 0.62	4.90 ± 0.41	5.43 ± 0.90	4.87 ± 0.86	5.68 ± 0.61
n-3/n-6	0.38 ± 0.03	0.39 ± 0.07	0.35 ± 0.04	0.34 ± 0.03	0.35 ± 0.03	0.38 ± 0.06	0.38 ± 0.03	0.39 ± 0.03	0.37 ± 0.03	0.35 ± 0.02	0.31 ± 0.02	0.28 ± 0.02	0.32 ± 0.05	0.32 ± 0.02
UFA/SFA	1.70 ± 0.12	1.98 ± 0.08	1.87 ± 0.19	2.03 ± 0.07	1.75 ± 0.16	1.94 ± 0.08	1.64 ± 0.10	1.71 ± 0.06	1.72 ± 0.09	1.84 ± 0.06	1.72 ± 0.11	2.16 ± 0.17	1.77 ± 0.16	1.92 ± 0.06
DBI	0.51 ± 0.02	0.51 ± 0.02	0.52 ± 0.03	0.53 ± 0.01	0.51 ± 0.02	0.52 ± 0.02	0.49 ± 0.02	0.47 ± 0.02	0.51 ± 0.02	0.51 ± 0.01	0.51 ± 0.02	0.53 ± 0.02	0.52 ± 0.03	0.51 ± 0.02
TACL	17.25 ± 0.05*	17.53 ± 0.07*	17.34 ± 0.05	17.55 ± 0.08	17.31 ± 0.04*	17.55 ± 0.07*	17.33 ± 0.05*	17.56 ± 0.07*	17.37 ± 0.04	17.53 ± 0.09	17.38 ± 0.04*	17.74 ± 0.10*	17.40 ± 0.07	17.65 ± 0.08

[a]An asterisk (*) indicates a significant difference between the fed and the fasted animals (*t*-test, Mann–Whitney U test, $P < 0.05$). For abbreviations see Table 1. The sums include also minor FA not presented in the table.

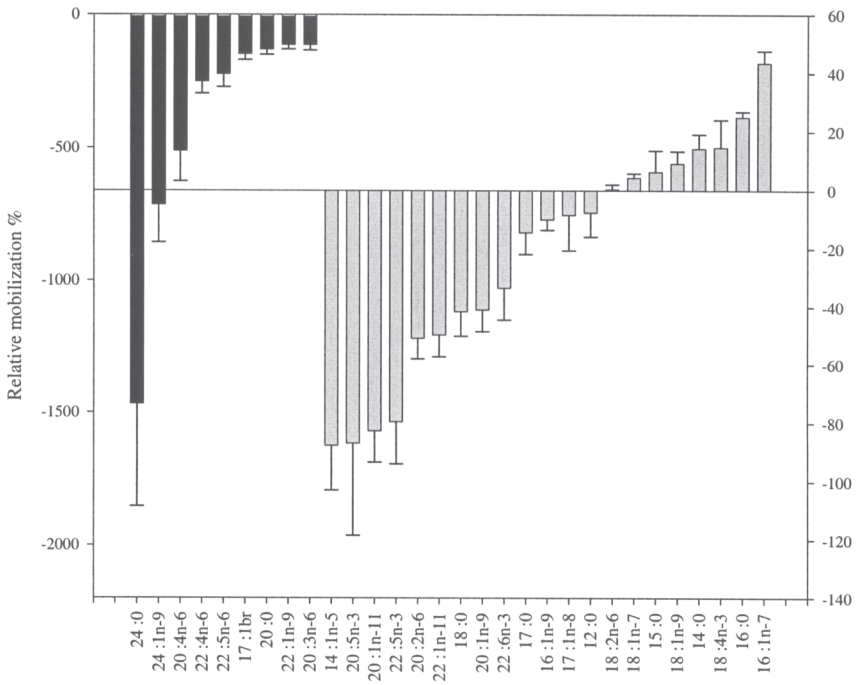

FIG. 1. *In vivo* relative mobilization of the most abundant FA in pooled adipose tissues of the American marten fasted for 2 d (mean + SE). Positive (+) values indicate that the proportion of a FA is lower compared with the fed control group, and negative (–) values signify that the proportion of a FA has increased compared with the fed control group. Black bars are plotted against the left Y-axis and gray bars against the right Y-axis.

MUFA, and, in particular, 2n-PUFA increased, while the proportions of 5n-PUFA decreased and the total percentage of the VLCFA increased. In the liver, the total proportion of SFA, 3n-, and 5n-PUFA was lower in the fasted animals. After 2 d of fasting, most FA were highly preserved in the fat depots (Fig. 1). The most important FA that had high relative mobilization were 16:1n-7, 16:0, 18:4n-3, and 14:0, whereas several long-chain MUFA and PUFA, such as 24:1n-9 and 20:4n-6, had mostly low relative mobilization, along with 14:1n-5, 17:0, 18:0, 20:0, and 24:0. Relative mobilization did not differ between the various iab and sc depots.

Relative mobilization of SFA correlated negatively with the chain length when all trunk WAT were analyzed as a whole (r_s = –0.327, $P < 0.01$; Fig. 2a). The same was observed in the n-9 MUFA (r_s = –0.350, $P < 0.01$; Fig. 2b) but not in the n-5, n-7, or n-11 MUFA. The influence of chain length on the relative mobilization of PUFA was tested by comparing PUFA with the same degree of unsaturation and position of the first double bond but with a different chain length. The difference was statistically significant in six out of eight pairs (16:2n-4 vs. 18:2n-4; 18:2n-6 vs. 20:2n-6; 18:3n-6 vs. 20:3n-6; 18:3n-3 vs. 20:3n-3; 18:4n-3 vs. 20:4n-3; and 20:4n-3 vs. 22:4n-3) in the way that the PUFA with the longer chain length had a lower mobilization rate (*t*-test, $P < 0.05$).

When SFA and corresponding MUFA with the same chain length were compared with each other, relative mobilization increased with Δ9-desaturation in four out of seven cases (16:0 vs. 16:1n-7; 18:0 vs. 18:1n-9; 19:0 vs. 19:1n-10, and 20:0 vs. 20:1n-11, *t*-test, $P < 0.05$; Fig. 2c). The influence of unsaturation on relative mobilization of PUFA was investigated by comparing PUFA with the same chain length and position of the first double bond but with a different double bond number. The difference was significant in three out of six pairs (18:3n-3 vs. 18:4n-3; 20:2n-6 vs. 20:3n-6; 20:3n-6 vs. 20:4n-6; Fig. 2d) in the way that the PUFA with the higher number of double bonds had the lower relative mobilization rate (*t*-test, $P < 0.05$).

The influence of positional isomerism on the relative mobilization of MUFA was tested by comparing MUFA of the same chain length but different position of the double bond. The difference was statistically significant in five out of seven pairs (*t*-test, $P < 0.05$) in the way that the MUFA with the double bond closer to the methyl end of the molecule had a lower (18:1n-5 vs. 18:1n-7; 20:1n-9 vs. 20:1n-11; and 22:1n-9 vs. 22:1n-11) or a higher mobilization rate (14:1n-7 vs. 14:1n-9 and 16:1n-7 vs. 16:1n-9). When comparing PUFA with the same chain length and unsaturation but with a different position of the first double bond, the difference was statistically significant in four out of six pairs (*t*-test, $P < 0.05$) in the way that the PUFA with the first double bond closer to the methyl end of the molecule had a lower (18:3n-3 vs. 18:3n-6; 18:2n-6 vs. 18:2n-7; and 22:5n-3 vs. 22:5n-6) or a higher mobilization rate (20:4n-3 vs. 20:4n-6).

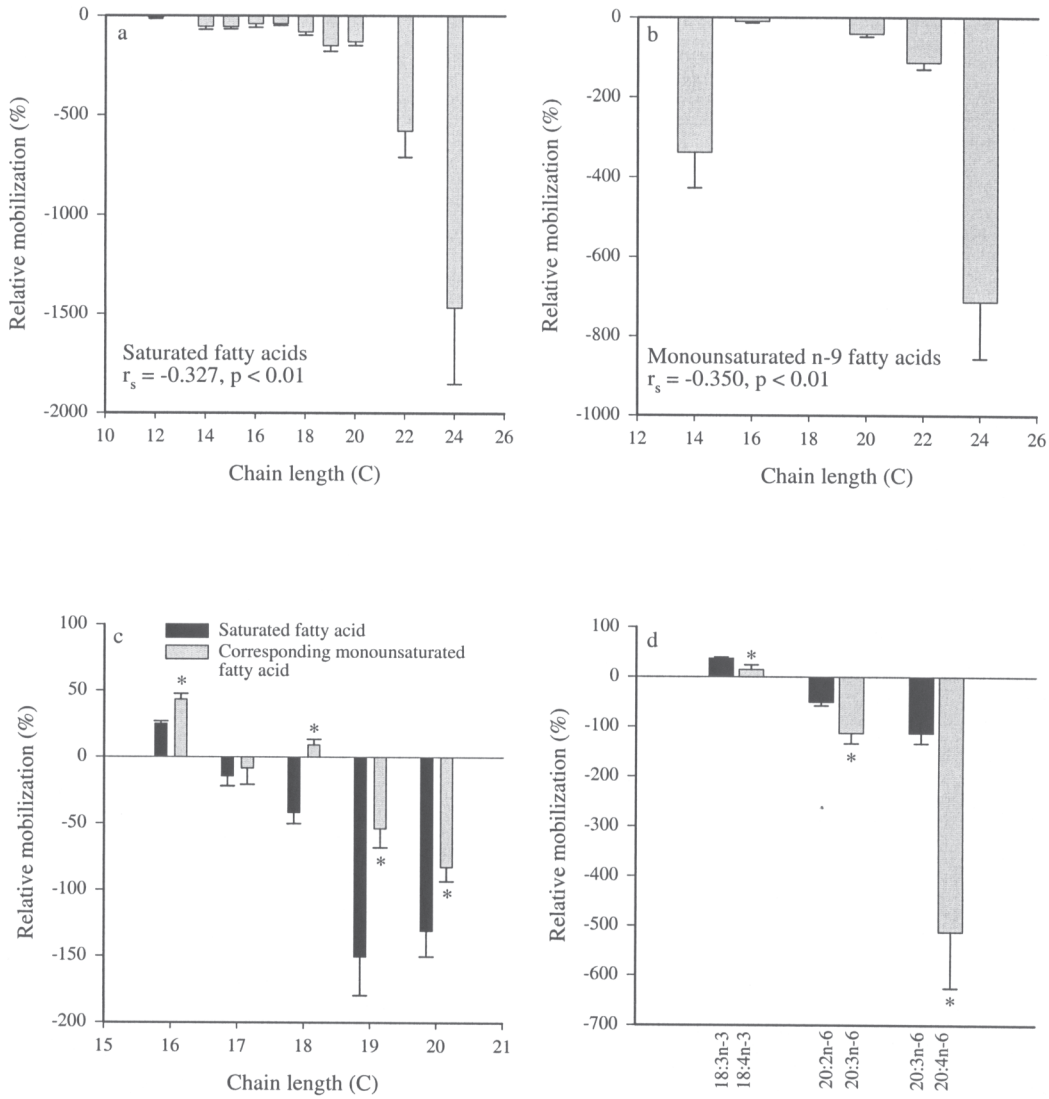

FIG. 2. Effects of FA structure on relative mobilization in the American marten after 2 d of fasting (all trunk white adipose tissues analyzed as a whole; mean + SE). (a) Relative mobilization of saturated FA of different carbon chain length. (b) Relative mobilization of n-9 monounsaturated FA of different carbon chain length. (c) The influence of Δ9-desaturation on relative mobilization. * = Significant difference in relative mobilization between saturated and monounsaturated FA (*t*-test, $P < 0.05$). (d) Effect of double bond number on relative mobilization of selected PUFA. * = Significant difference in relative mobilization between PUFA with different degrees of unsaturation (*t*-test, $P < 0.05$).

DISCUSSION

Body fat content and rate of weight loss. The rate of BM loss in the fasted American marten was 13.4% or 6.7% d^{-1}. This is slightly higher than the BM loss of 4.8% d^{-1} observed previously after a 5-d fasting period (12). At the same time, the BMI of the fasted marten of this study decreased by 18.5%. The total fat percentage of the marten was very low, approximately 2.7% for the fed animals and only a little more than 2% for the fasted animals. The data of the fed control animals fit quite well the previous report of Buskirk and Harlow (11). They measured a 2.4% body fat content in skinned American marten in Alaska, whereas in Wyoming the whole body fat content was approximately 5.6% in winter. The rate of BM loss in the marten was relatively high compared with a close relative, the farmed sable, which lost about 16% or 4.0% d^{-1} of its BM during a 4-d wintertime fasting period (Mustonen, A.-M., Puukka, M., Saarela, S., Paakkonen, T., Aho, J., and Nieminen, P., unpublished data). The sable had an 8.0% body fat content. The body fat percentage of the captive marten of this study was even more different from the farmed American mink, which can have a body fat content as high as 35–38% in the winter (15). Fasted mink lose weight at a rate of approximately 3.0% after a 2-d fasting period, which is about 4.5 times less than the weight loss rate of the marten was.

The smaller initial body fat percentage compared with the sable or the mink could be an explanation of the more rapid weight loss of the marten. Marten have been previously fasted for 5 d with fat as the principal source of metabolic energy (12). Yet the use of proteins is already quite significant at this point of fasting. It is conceivable that some body proteins could have been lost during the fasting procedure of the present study, as the total BM of the fasted marten decreased by 113 g, of which fat accounted for approximately 8.2 g. A major part of the BM loss probably consisted of intestinal contents, but also body proteins and carbohydrates could have contributed to the observed BM loss. However, plasma urea and ammonia levels as well as plasma, liver, and muscle protein concentrations remained stable during fasting while the liver, muscle, and kidney glycogen stores decreased (Nieminen, P., Rouvinen-Watt, K., and Mustonen, A.-M., unpublished data). These data indicate that the marten were still within phase II of fasting at the end of the experiment.

As an additional result, the clearly positive correlation between the BMI and the total fat mass or the body fat percentage indicates that the BMI can be used as an accurate and easily measured tool to approximate the body condition and fat content of American marten. To determine the condition of an individual marten, this can be supplemented by measuring the om fat mass (22). In fact, the correlation between the calculated BMI and the different fat depots was the most significant between the BMI and the om fat mass ($r_s = 0.828$, $P < 0.01$). The observation that the marten in the fasted group were physically active, many of them exhibiting stereotypical behavior and signs of stress, may be quite relevant to their rapid rate of body mass loss. This is most likely due to increased energy expenditure caused by accelerated locomotor activity as observed previously in fasted mink (23). In a similar manner, it has been observed that physical activity increases in the least weasel (*Mustela nivalis*) due to food deprivation (24).

Site-specific FA composition of the American marten. Generally, the FA composition in the sc and iab depots of the American marten was very similar to previous findings on other mustelids, the sable and the mink (25,26; Nieminen, P., and Mustonen, A.-M., unpublished data; Nieminen, P., Käkelä, R., Pyykönen, T., and Mustonen, A.-M., unpublished data). The most abundant FA in the fat depots of the marten were 14:0, 16:0, 18:0, 16:1n-7, 18:1n-9, and 18:2n-6. The proportions of total MUFA and PUFA in the trunk fat depots (42–43% and 22–23%, respectively) were closer to those observed previously in the farmed sable (40–45% and 20–25%) than to those in the farmed mink (50–55% and 10%). In this respect, the American marten is very similar to its Siberian relative, the sable, wheras both differ from the more distantly related mink. However, it must be recalled that the FA composition of the diets affecting the tissue FA percentages was probably dissimilar between the species of these experiments and could have caused some of the observed differences.

Proportions of MUFA and PUFA were much higher in the extremities than in the trunk of the marten. This was especially evident in the tip of the tail, with many n-3 PUFA, such as 20:3n-3, 20:5n-3, and 22:6n-3, in higher proportions than in the trunk. Furthermore, there was a simultaneous increase in the sum of n-3 PUFA in all the extremity tissues. The longer-chain 20- and 22-carbon PUFA were detected at higher proportions in the paws. In addition, the indices of unsaturation, the Δ9-DI, DBI and UFA/SFA ratio, were higher in the extremities, especially in the tail tip.

Previously, the FA composition of the extremities of mustelids has been studied in the mink and the sable (Mustonen, A.-M., Käkelä, R., and Nieminen, P., unpublished data; Mustonen, A.-M., and Nieminen, P., unpublished data). In this respect, the American marten is quite similar to the mink, as both species have decreased proportions of SFA and increased desaturation in the periphery. The differences between the fat depots of the trunk and the extremities are less pronounced in the sable, yet sables also have increased proportions of n-3 PUFA and VLCFA in their peripheral tissues. In general, the m.p. of mammalian fats are approximately 30–40°C, whereas the least insulated parts of the body, such as paw pads and the tail tip, may cool significantly more, down to 0°C in some conditions. The higher proportion of UFA in the periphery is probably crucial to prevent the solidification of lipids to maintain the fluidity of cell membranes. In fact, similar FA gradients have been detected from other boreal and arctic mammalian species, such as the European otter (*Lutra lutra*; 27), the blue fox (*Alopex lagopus*; 26), and beavers (*Castor fiber* and *C. canadensis*; 28). For instance, the adipose depots under the footpads of the otter have significantly more MUFA than the fat tissues of the trunk (27). Also, in the American marten, this phenomenon must be taken as an adaptation to the cold winter environment the species experiences in its natural habitat.

Effects of food deprivation on the relative mobilization of FA. In the fat depots of the trunk, the 2-d fasting period induced significant changes in the FA composition. In the SFA the proportions of long-chain FAs, especially 18:0, 20:0, and 22:0, increased with very few exceptions in most of the WAT depots, whereas the proportion of 24:0 increased especially in the iab fat. Simultaneously, the proportions of 16:0 decreased in all the studied fat tissues. Furthermore, the percentages of 18:3n-3 decreased in both pooled sc and iab fats, and the proportion of 18:4n-3 decreased in the iab fat, whereas the percentages of many n-6 PUFA, such as 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6 increased. This caused an elevation in the sum of n-6 PUFA in both pooled sc and iab fats. At the same time, the proportion of 22:6n-3, an important FA for biological membranes (29), was unaffected by fasting. The conversion of various n-3 PUFA into the biologically important 22:6n-3 can be an explanation to the stable proportion of 22:6n-3 despite of fasting.

An increase in the proportion n-6 PUFA would be considered undesirable from the point of view of the human cardiovascular system as it causes harmful alterations in eicosanoid production (30). As the proportions of n-3-derived prostaglandins and eicosanoids decrease, unwanted proinflammatory changes in the immune response as well as cardiac arrhythmias and increased platelet aggregation may ensue. However, despite

this general effect of fasting on n-6 PUFA, the effects of positional isomerism on the relative mobilization of PUFA were inconsistent and no definite conclusions on the effects of fasting on n-3 and n-6 PUFA can be drawn from these data. The apparent increase in the proportion of n-6 PUFA can be caused by the analysis of FA from total lipids. There could have been a relative increase in the proportion of membrane phospholipids in comparison with storage TAG during fasting in the marten with a low body fat content. For instance, the increased proportion of phospholipids can be an explanation for the observed increases in 18:0 and 20:4n-6 in the fasted animals.

After 2 d of fasting, most of the relatively abundant FA were preserved in the fat depots. The anatomically different adipose tissues of the marten were very similar in this respect, as no significant differences in the mobilization rates of individual FA were found between the different WAT depots. Likewise, rats (*Rattus norvegicus*; 31) do not experience fat depot-associated differences in relative mobilization. The FA that were preferentially mobilized by the fasted marten were 16:1n-7; short-chain SFA, especially 14:0 and 16:0; 18:1n-9; and, in some of the fat depots, particular n-3 PUFA (18:4n-3).

The structure of FA may partly dictate the selectivity of FA mobilization. In the case of the American marten, short-chain FA were more readily mobilized over long-chain FA in all FA classes (SFA, MUFA, and PUFA). Shorter FA molecules are hypothesized to be situated peripherally in lipid droplets and can thus be more easily accessible to lipases (32). As a result, the proportion of longer-chain FA increases as the shorter-chain FA are mobilized for energy production. The same could be observed in the negative correlation between relative mobilization and chain length in all the fat depots of the trunk: the longer the carbon chain, the more obvious the preservation of a FA in an adipose tissue depot.

For a particular chain length, relative mobilization of FA increases with the number of double bonds in the rat (31). The same could be observed in the marten, as the mobilization of MUFA was higher than that of their corresponding SFA in 71% of the studied cases. However, in PUFA the effect was the opposite, as relative mobilization decreased with the number of double bonds in 50% of the cases. In this study, the effects of positional isomerism on relative mobilization were quite vague. This suggests that relative mobilization of FA during fasting is not as straightforward in all mammalian species as suggested by previous *in vitro* (32) and *in vivo* studies (31).

Ecophysiological implications. According to Buskirk (22), the American marten has limited fat reserves in the winter (2.4% of skinned carcass) and the om fat mass is considered the best indicator of body energy content. This would indicate that marten do not have enough stored body fat available to fast for long periods when foraging is impossible, e.g., during severe weather. To cope with this, marten may use microhabitats to avoid extremely cold temperatures. Furthermore, Thompson (33) suggests that marten reduce energy loss during winter by reducing activity, by eating large prey, and by being active during the warmest part of the day.

Despite its very low body adiposity, the American marten has adaptations to withstand short periods of food deprivation encountered in nature especially in the winter. According to Buskirk (22), if a marten had a body fat content of 2.37%, approximately the same as in the fed marten of this study, it would take 70 h for the animal to metabolize its fat reserves completely, putting the marten of this experiment still within the phase II of fasting. In fact, the marten were able to mobilize all their principal adipose tissue depots of the trunk during food deprivation. The FA mobilized were almost the same in sc and iab fats, indicating that as a terrestrial species the American marten is not very dependent on sc fat for insulation and can use it as an energy reserve for intermittent fasting. The principal FA mobilized were relatively short-chain SFA (14:0 and 16:0) and MUFA (16:1n-7 and 18:1n-9), and in the case of the iab fat, n-3 PUFA, especially 18:3n-3 and 18:4n-3. In contrast to this, the FA composition of the extremities was very resistant to fasting and contained significantly more long-chain PUFA than the depots of the trunk. This is probably an adaptation to maintain cell membrane fluidity and prevent freezing and solidification of these parts of the body most susceptible to cooling and frostbite.

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Effects of Conjugated Linoleic Acid and Troglitazone on Lipid Accumulation and Composition in Lean and Zucker Diabetic Fatty (*fa/fa*) Rats

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ABSTRACT: Dietary CLA has been shown to enhance glucose tolerance in several animal models, but in mice it induces insulin resistance and lipodystrophy. In this study, the effects of 2 wk of diet supplementation with either 1.5% CLA or 0.2% troglitazone (TZD), an insulin-sensitizing thiazolidinedione, on glucose tolerance, lipid accumulation, and composition of both lean and Zucker diabetic fatty (*fa/fa*; ZDF) rats were examined. Compared with lean rats, which maintained normal glucose tolerances after 2 wk of feeding regardless of diet, ZDF rats fed a control diet (CON) had significantly worsened glucose tolerance. ZDF rats fed CLA and TZD diets, however, maintained normal glucose tolerances. In contrast to the significantly elevated lipid levels in ZDF rats fed the CON diet, concentrations of plasma FFA and TG in ZDF rats fed CLA and TZD diets were normalized. A similar reduction of plasma lipid levels was observed in lean rats fed CLA and TZD compared with lean rats fed the CON diet. Although ZDF CON rats developed significant hepatic steatosis, both CLA- and TZD-fed rats had hepatic TG levels similar to those of lean rats. Both lean and ZDF rats fed the CLA diet had reduced adipose mass compared with respective genotype controls; however, TZD had no effect. Ratios of 16:1/16:0 and 18:1/18:0 FA, surrogate markers for stearoyl-CoA desaturase-1 (SCD-1) activity, were reduced in livers of ZDF rats fed CLA and TZD diets. These results show that, like TZD, CLA normalizes glucose tolerance and plasma lipids and also improves hepatic steatosis and FA composition in ZDF rats. The effects of CLA and TZD on hepatic lipid composition suggest that the effects of these two agents on glucose tolerance may be associated with a reduction in SCD-1.

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CLA refers to a group of PUFA that exist as positional and stereoisomers of octadecadienoic acid (18:2). CLA is found naturally in foods derived from ruminants, such as beef, lamb, and dairy products, and in supplement form (e.g., Tonalin™). Due to the potential health benefits of CLA, a great deal of research has been devoted to this group of PUFA. CLA acts as an anticarcinogenic (1) and anti-atherosclerogenic agent, as a modulator of immune function and body composition, and as a possible antidiabetic agent (2). In the treatment of diabetes, CLA improves glucose tolerance and insulin sensitivity, as well as fasted glucose and insulin levels in several rodent models of obesity and diabetes (3–7). Further, CLA reduces plasma FFA (3,5–8) and plasma (3,6–8) and hepatic (9) TG concentrations, which are thought to contribute to insulin resistance and subsequent increased hepatic glucose production (10). The decrease in hepatic TG and altered lipid metabolism by CLA may be attributed, in part, to the reduction of stearoyl-CoA desaturase-1 (SCD-1), a rate-limiting enzyme in the desaturation of palmitoyl- (16:0) and stearoyl-CoA (18:0) FA that is essential to TG formation. Previous research has shown that CLA reduces SCD-1 mRNA expression and activity in several different *in vitro* and *in vivo* models (11–14). Nevertheless, the mechanism and the relative efficacy of CLA to improve glucose tolerance, insulin sensitivity, and the associated lipid metabolism have not been fully elucidated.

Similarly, treatment with thiazolidinediones, insulin-sensitizing agents used in type 2 diabetes therapy, lowers fasting glucose and insulin concentrations, corrects plasma lipid levels (15), and reduces hepatic TG levels (16). Thiazolidinediones also have been shown to decrease SCD-1 expression and activity (17,18), which may contribute to the reductions in lipid levels. Additionally, thiazolidinediones function as a high-affinity ligand for the nuclear receptor peroxisome proliferator activated receptor-γ (PPARγ). PPARγ, in turn, stimulates the differentiation, proliferation, and lipid accumulation of adipocytes, thereby increasing adiposity (19). The induction of adipocyte differentiation promotes lipid accumulation in adipose tissue and prevents lipid accumulation in peripheral tissues such as liver, muscle, and pancreas. The ability of thiazolidinediones to prevent steatosis in nonadipose tissues, as well as other indirect mechanisms, may contribute to the activity of thiazolidinediones as insulin sensitizers (20).

Our group reported that, following 11 d on experimental diets, Zucker diabetic fatty (ZDF) rats fed diets supplemented with either CLA or a thiazolidinedione had similar fasting glucose levels as lean rats, whereas ZDF rats fed a control diet had elevated fasting glucose levels (5). Because glucose metabolism and insulin sensitivity are associated with reduced steatosis (21) and decreased SCD-1 activity (22,23), the objective of this study was to compare the effects of CLA and a thiazolidinedione on lipid composition and metabolism in the liver. The impact of CLA on glucose tolerance, plasma lipid levels, and hepatic lipid accumulation, composition, and SCD-1 activ-

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Abbreviations: AUC, area under curve; CON, control; PPARγ, peroxisome proliferator activated receptor-γ; SCD-1. stearoyl-CoA desaturase-1; TZD, troglitazone; ZDF, Zucker diabetic fatty (Gmi-*fa/fa*).

ity was compared with this known type 2 diabetes agent in both lean and ZDF rats, as many of the antidiabetic effects of CLA and thiazolidinediones are similar.

MATERIALS AND METHODS

Materials. Diet components were obtained from Dyets, Inc. (Bethlehem, PA). The CLA oil (Pharmanutrients, Inc., Lake Bluff, IL) was ~90% CLA with the following composition: 42% *c*9, *t*11- and *t*9, *c*11-CLA; 43.5% *t*10, *c*12-CLA; 1% *c*9, *c*11-CLA; 1% *c*10, *c*12-CLA; 1.5% *t*9, *t*11- and *t*10, *t*12-CLA; 0.5% linoleate; 5.5% oleate; and 5% unidentified. The use of this mixed isomer form of CLA is most relevant regarding the impact of CLA on the management of type 2 diabetes as this source of CLA is currently available commercially for use in humans (e.g., Tonalin™). The thiazolidinedione troglitazone (TZD) was obtained from Parke-Davis (Ann Arbor, MI).

Animals and experimental diets. Six-week-old, male, obese, Zucker diabetic fatty (ZDF/Gmi-*fa/fa*) rats (n = 24) and lean littermates (ZDF/Gmi-+/?; n = 12) were purchased from Genetic Models, Inc. (Indianapolis, IN). Prior to beginning experimental diets, rats were determined to be normoglycemic by a fasting glucose measurement. Rats were then randomized by genotype and body weight and assigned to one of three isocaloric, modified AIN-76 diets containing 6.5% fat for 14 d (from 8–10 wk of age). Corn oil (5%) was used in all diets, and research-grade, tocopherol-stripped lard was used to balance caloric density so that diets contained either 5% corn oil/1.5% lard/no CLA (CON); 5% corn oil/1.5% CLA (CLA); or 5% corn oil/1.5% lard/0.2% TZD. Each diet group contained 8 ZDF rats and 4 lean rats. All procedures were in accordance with institution guidelines and approved by the Purdue Animal Care and Use Committee. A glucose tolerance test was conducted on day 11 of dietary intervention after an overnight (16 h) fast as previously reported (5). Glucose areas under the curve (AUC) are expressed as positive incremental AUC calculated using the trapezoidal rule (24). After 14 d on experimental diets, rats were euthanized by CO_2 and cervical dislocation. Blood was collected immediately in heparinized test tubes for plasma analyses as described below. Liver and adipose tissues were harvested, weighed, and immediately frozen and stored at –80°C.

Analysis of TG and FFA. Plasma FFA were determined using a colorimetric kit (NEFA C; Wako Chemicals, Richmond, VA). Lipids were extracted from tissues with chloroform/methanol (2:1, vol/vol) and 0.2 vol 0.88% KCl. To measure TG concentrations, lipid extracts were further processed as previously described by Denton and Randle (25) with alterations by Frayn and Maycock (26). In brief, phospholipids were removed with silicic acid, and the remaining lipids were saponified with 80% KOH/ethanol (5:95, vol/vol). Addition of 0.15 M $MgSO_4$ stopped the saponification; and after centrifugation, the supernatants, as well as plasma, were analyzed for TG by colorimetric enzymatic hydrolysis (Triglyceride GPO-Trinder reagents; Sigma, St. Louis, MO).

FA analyses. Lipid extracts were separated into neutral lipids and phospholipids by the silica column procedure described by Hamilton and Comai (27). In brief, neutral and phospholipids were separated by PrepSep Si columns (Fisher Scientific, Fair Lawn, NJ) by eluting extracts with methyl *tert*-butyl ether/acetic acid (100:0.2, vol/vol) and methyl *tert*-butyl ether/methanol/ammonium acetate (pH 8.6) (5:8:2, by vol), respectively. FAME of the fractions were prepared by incubating the fractions with 1,1,3,3-tetramethylguanidine at 100°C and analyzed by GC using a 30-m Omegawax 320 capillary column (Supelco, Sigma-Aldrich, St. Louis, MO). The helium flow rate was 30 mL/min, and the oven temperature was programmed to start at 175°C for 4 min and increase to 220°C at a rate of 3°C/min.

Statistical analyses. Data are expressed as least square means ± SE. Effects of genotype (lean and ZDF) and diet (CON, CLA, and TZD) were analyzed by two-way ANOVA using the MIXED procedure of Statistical Analysis System (SAS V8; Cary, NC). Differences of $P < 0.05$ were considered significant.

RESULTS

Glucose tolerance. A glucose tolerance test was administered after 11 d on experimental diets. There were no differences in the AUC among any of the lean diet groups. Although ZDF rats fed the CON diet had significantly worsened glucose tolerance as indicated by elevated AUC, ZDF rats fed CLA or TZD diets maintained an AUC similar to that of lean rats (Fig. 1).

Plasma FFA and TG levels. Plasma FFA concentrations were reduced by CLA and TZD treatment in both lean and ZDF rats. Plasma FFA levels were similar between lean and ZDF rats fed the CON diets, but were significantly reduced in lean

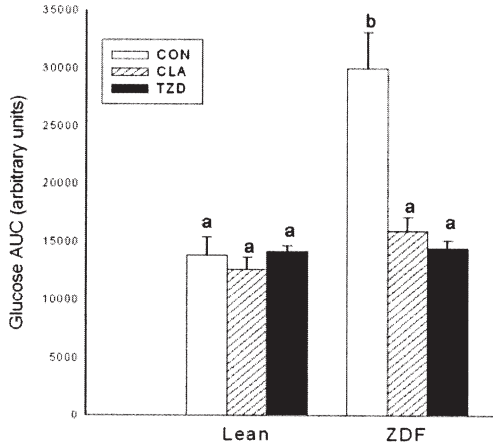

FIG. 1. Effect of dietary CLA and troglitazone (TZD) on glucose tolerance test in lean and Zucker diabetic fatty (ZDF) rats. Rats were fed either a control (CON; open bars), 1.5% CLA (hatched bars), or 0.2% TZD (closed bars) diet for 14 d. A glucose tolerance test was administered on day 11 of experimental diets after a 16-h fast as described in the Materials and Methods section. Values represent least squares mean (LSM) ± SE. Values with different superscripts are significantly different ($P < 0.05$). AUC, area under the curve.

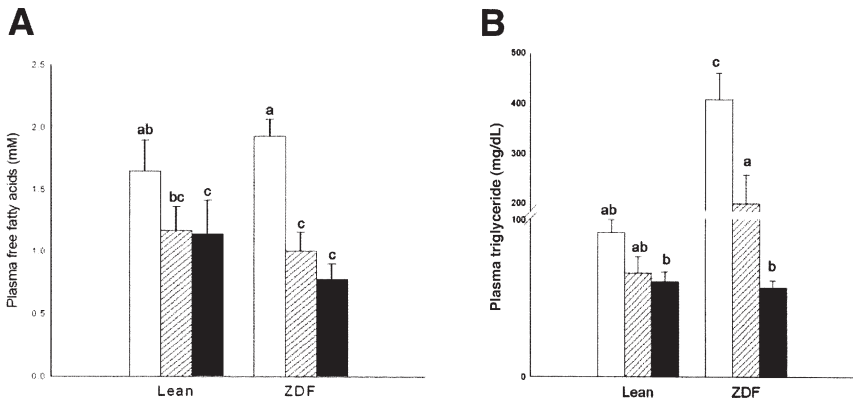

FIG. 2. Effect of dietary CLA and TZD on plasma (A) FFA and (B) TG levels in lean and ZDF rats. Rats were fed either a control (CON; open bars), 1.5% CLA (hatched bars), or 0.2% (closed bars) diet for 14 d. FFA and TG levels were measured as described in the Materials and Methods section. Values represent LSM ± SE. Values with different superscripts are significantly different ($P < 0.05$). For abbreviations see Figure 1.

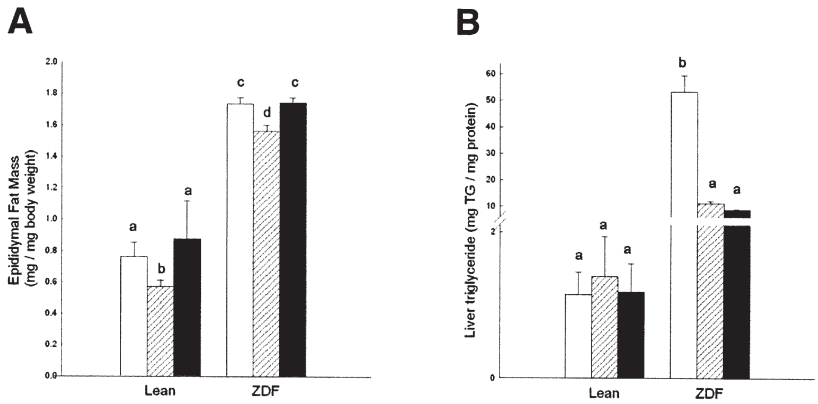

FIG. 3. Effect of dietary CLA and TZD on (A) epididymal fat mass and (B) liver TG levels in lean and ZDF rats. Rats were fed either a control (CON; open bars), 1.5% CLA (hatched bars), or 0.2% TZD (closed bars) diet for 14 d. TG levels were measured as described in Materials and Methods section. Values represent LSM ± SE. Values with different superscripts are significantly different ($P < 0.05$). For abbreviations see Figure 1.

TZD and in ZDF CLA and TZD rats. The plasma FFA concentration in lean rats fed CLA was similar to the reduced levels but was not statistically lower compared with lean CON (Fig. 2A). Plasma TG levels did not differ among diet groups of lean rats (Fig. 2B). Although the plasma TG concentrations of ZDF rats fed the CON diet were significantly elevated compared with lean rats fed the CON diet, both CLA and TZD treatments in ZDF rats reduced plasma TG. Additionally, lean and ZDF rats fed the TZD diet had significantly lower plasma TG levels than ZDF rats fed the CLA diet.

Epididymal fat mass and liver TG concentrations. Regardless of dietary group, ZDF rats had significantly more epididymal fat mass than all the lean diet groups following 14 d on experimental diets (Fig. 3A). Rats fed the CLA diet, whether the lean or ZDF group, had lower epididymal fat mass than the CON and TZD-fed rats within their respective genotype group. Liver TG concentrations in lean rats were not altered by diet (Fig. 3B). ZDF rats fed the CON diet had significantly elevated levels of TG in their livers, but CLA and TZD treatment maintained TG levels in ZDF rats similar to those of lean rats.

Liver FA composition. As expected, compared with rats fed the CON and TZD diets, rats fed the CLA diet, regardless of genotype, accumulated greater amounts of both isomers in both the neutral lipid and phospholipid fractions of the liver (Tables 1, 2). In both lipid fractions of the liver, all ZDF rats had significantly reduced levels of arachidonic acid (20:4) compared with lean animals. While dietary treatment had no effect on arachidonic acid levels in lean rats, arachidonic acid levels were significantly higher in ZDF rats fed CLA and TZD (Tables 1, 2). Ratios of palmitoleate (16:1)/palmitate (16:0) and oleate (18:1)/stearate (18:0) were used as an index of SCD activity. Compared with lean rats, all ZDF rats had elevated SCD in-

TABLE 1
Effects of Dietary CLA and TZD on FA Composition in the Neutral Lipid Fraction of the Livers of Lean and ZDF Rats

	Lean			ZDF		
FA[a] (%)	CON	CLA	TZD	CON	CLA	TZD
14:0	0.28 ± 0.05[a]	0.32 ± 0.06[a]	0.24 ± 0.05[a]	1.55 ± 0.05[d]	1.17 ± 0.05[c]	0.80 ± 0.05[b]
16:0	19.88 ± 0.60[a]	22.40 ± 0.70[b]	23.29 ± 0.60[b]	40.94 ± 0.60[e]	36.02 ± 0.60[d]	33.60 ± 0.60[c]
16:1	1.17 ± 0.30[a]	1.36 ± 0.35[a]	1.48 ± 0.30[a]	8.65 ± 0.30[d]	6.11 ± 0.30[c]	5.15 ± 0.30[b]
18:0	20.94 ± 0.51[c]	20.52 ± 0.59[c]	20.26 ± 0.51[c]	7.25 ± 0.51[a]	14.42 ± 0.51[b]	14.87 ± 0.51[b]
18:1 n-9	7.95 ± 0.84[a]	8.82 ± 0.97[a]	9.40 ± 0.84[a]	28.53 ± 0.84[c]	19.89 ± 0.84[b]	18.66 ± 0.84[b]
18:1 n-7	3.51 ± 0.11[c]	2.77 ± 0.13[b]	2.69 ± 0.11[b]	2.83 ± 0.11[b]	2.69 ± 0.11[b]	2.11 ± 0.11[a]
18:2	18.95 ± 0.67[d]	15.58 ± 0.77[c]	15.18 ± 0.67[c]	5.71 ± 0.67[a]	6.32 ± 0.67[a]	8.54 ± 0.67[b]
18:3	0.15 ± 0.04[a]	0.21 ± 0.05[a,b]	0.30 ± 0.04[b]	0.19 ± 0.04[a,b]	0.24 ± 0.04[a,b]	0.60 ± 0.04[c]
*c*9,*t*11 CLA	0.00 ± 0.14[a]	0.72 ± 0.17[b]	0.00 ± 0.14[a]	0.03 ± 0.14[a,b]	0.21 ± 0.14[a,b]	0.05 ± 0.14[a]
*t*10,*c*12 CLA	0.00 ± 0.09[a]	0.40 ± 0.11[b]	0.00 ± 0.09[a]	0.00 ± 0.09[a]	0.11 ± 0.09[a,b]	0.01 ± 0.09[a]
20:1	0.21 ± 0.02[b]	0.12 ± 0.02[a]	0.09 ± 0.02[a]	0.07 ± 0.02[a]	0.09 ± 0.02[a]	0.07 ± 0.02[a]
20:4	26.95 ± 1.02[c]	26.79 ± 1.18[c]	27.08 ± 1.02[c]	4.26 ± 1.02[a]	12.73 ± 1.02[b]	15.54 ± 1.02[b]
16:1/16:0	0.06 ± 0.01[a]	0.06 ± 0.01[a]	0.06 ± 0.01[a]	0.21 ± 0.01[c]	0.17 ± 0.01[b]	0.15 ± 0.01[b]
18:1/18:0	0.38 ± 0.19[a]	0.43 ± 0.22[a]	0.47 ± 0.19[a]	4.02 ± 0.19[c]	1.38 ± 0.19[b]	1.27 ± 0.19[b]

[a]FA expressed as a percentage of total reported FA. Values are least squares mean (LSM) ± SE. Different superscripts within a row indicate significant differences ($P < 0.05$). TZD, troglitazone; ZDF, Zucker diabetic fatty.

dices in both lipid fractions of the liver; however, the increase was partially rescued by both CLA and TZD treatment in the ZDF rats (Tables 1, 2).

DISCUSSION

Of the prospective health benefits of CLA, the potential antidiabetic effects may be the most controversial due to conflicting data. This report agrees with others that have shown CLA attenuates insulin resistance and glucose intolerance in diabetic or obese rat models (3–6,28). However, other groups have shown that CLA induces insulin resistance and hyperglycemia primarily in mouse models (7,29–31). In mice, insulin resistance develops in parallel with severe lipoatrophy and hepatic steatosis (29). Consequently, the ablation of adipose tissue caused by CLA results in lipid accumulation in peripheral tissues, such as liver, as well as decreases in adipocytokines, such as leptin and adiponectin (29,32,33), all of which may contribute to insulin resistance. Tsuboyama-Kasaoka *et al.* (34) demonstrated that feeding CLA with a high-fat diet partially preserves adipose mass and prevents hyperinsulinemia and hepatomegaly. These results suggest that the ability of CLA to enhance insulin sensitivity is dependent on maintaining a certain level of adipose mass and therefore preventing a marked reduction in adipocytokines and lipid accumulation in peripheral tissues, especially the liver.

In the present study, the effects of dietary CLA and TZD were compared in obese (Gmi-*fa/fa*) and lean (Gmi-+/?) ZDF rats. In contrast to lean rats that are nonobese and otherwise phenotypically "normal," ZDF rats have a mutated, nonfunctional leptin receptor (35). ZDF rats are therefore leptin resistant, obese, and develop hyperlipidemia, hyperleptinemia, insulin resistance, and hyperglycemia in a pattern similar to type 2 diabetes (36,37). As in mice and other animal models (38,39),

TABLE 2
Effects of Dietary CLA and TZD on FA Composition in the Phospholipid Fraction of the Livers of Lean and ZDF Rats

	Lean			ZDF		
FA[a] (%)	CON	CLA	TZD	CON	CLA	TZD
14:0	0.25 ± 0.05[a,b]	0.23 ± 0.06[a,b]	0.14 ± 0.05[a]	0.70 ± 0.05[c]	0.74 ± 0.05[c]	0.37 ± 0.05[b]
16:0	26.16 ± 1.30[a]	26.30 ± 1.50[a]	26.06 ± 1.30[a]	35.60 ± 1.30[b]	35.60 ± 1.50[b]	35.70 ± 1.30[b]
16:1	0.60 ± 0.28[a]	0.65 ± 0.32[a]	0.80 ± 0.28[a]	4.13 ± 0.28[c]	3.31 ± 0.28[b,c]	2.69 ± 0.28[b]
18:0	24.82 ± 1.56[a,b]	25.20 ± 1.80[a,b]	23.17 ± 1.56[a,b]	22.65 ± 1.56[a,b]	25.49 ± 1.56[b]	20.21 ± 1.56[a]
18:1 n-9	4.90 ± 0.88[a]	4.96 ± 1.02[a]	7.52 ± 0.88[a]	13.92 ± 0.88[c]	12.81 ± 0.88[b]	10.16 ± 0.88[b]
18:1 n-7	3.21 ± 0.25[b,c]	2.50 ± 0.29[a]	2.59 ± 0.25[a,b]	2.95 ± 0.25[a,b,c]	3.38 ± 0.25[c]	2.30 ± 0.25[a,b]
18:2	14.29 ± 0.73[c]	11.53 ± 0.84[b]	11.19 ± 0.73[b]	8.05 ± 0.73[a]	7.76 ± 0.73[a]	7.71 ± 0.73[a]
18:3	0.20 ± 0.04[a]	0.14 ± 0.05[a]	0.34 ± 0.04[b]	0.30 ± 0.04[a,b]	0.32 ± 0.04[a,b]	0.73 ± 0.04[c]
*c*9,*t*11 CLA	0.28 ± 0.05[b,c]	0.61 ± 0.06[c]	0.31 ± 0.05[b]	0.00 ± 0.05[a]	0.29 ± 0.05[b]	0.08 ± 0.05[a]
*t*10,*c*12 CLA	0.43 ± 0.05[c]	0.75 ± 0.06[d]	0.51 ± 0.05[c]	0.00 ± 0.05[a]	0.26 ± 0.05[b]	0.00 ± 0.05[a]
20:1	0.31 ± 0.04[b,c]	0.28 ± 0.05[b]	0.45 ± 0.04[c]	0.22 ± 0.04[a,b]	0.22 ± 0.04[a,b]	0.15 ± 0.04[a]
20:4	24.56 ± 1.23[c]	26.85 ± 1.42[c]	26.93 ± 1.23[c]	11.49 ± 1.23[a]	18.73 ± 1.23[b]	19.90 ± 1.23[b]
16:1/16:0	0.02 ± 0.01[a]	0.02 ± 0.01[a]	0.03 ± 0.01[a]	0.12 ± 0.01[c]	0.08 ± 0.01[b]	0.08 ± 0.01[b]
18:1/18:0	0.20 ± 0.04[a]	0.20 ± 0.05[a,b]	0.33 ± 0.04[b]	0.62 ± 0.04[d]	0.51 ± 0.04[c,d]	0.50 ± 0.04[c]

[a]FA expressed as a percentage of total reported FA. Values are LSM ± SE. Different superscripts within row indicate significant differences ($P < 0.05$). For abbreviations see Table 1.

both lean and ZDF rats fed CLA had significantly reduced adipose mass. However, the reduction in adipose in either genotype was not sufficient to induce lipoatrophy and subsequent hepatic steatosis as observed in mice (29,34). Although liver TG were significantly elevated in ZDF CON rats, liver TG of ZDF rats fed CLA were similar to lean rats. TZD, a PPARγ agonist, did not affect adipose mass in this 2-wk study but, consistent with another report (16), reduced liver TG. Notably, liver TG levels in lean rats were not affected by either treatment. These results support previous reports that CLA reduced adipose mass and hepatic steatosis in ZDF and Zucker rats (4,9,40). Furthermore, CLA was as effective as TZD in lowering liver TG and did not induce hepatic steatosis in lean rats. The prevention and reduction of hepatic steatosis is important for the ability of CLA to improve glucose tolerance, as hepatic steatosis contributes to insulin resistance and increased hepatic glucose production. Additionally, despite ZDF rats being leptin resistant, a condition that contributes to hepatic steatosis (41), CLA was able to prevent hepatic steatosis. Nagao *et al.* (9) showed that CLA increased adiponectin, an insulin-sensitizing adipocytokine, and suggested that the increase may have prevented the development of hepatic steatosis. Although adiponectin levels were not measured in this study, others have shown that CLA enhances adiponectin levels in ZDF rats (28).

The decrease in liver TG by CLA treatment in ZDF rats may also be partially attributed to the reduction of SCD-1. SCD-1 is important in lipid metabolism as it catalyzes the Δ-9 desaturation predominantly of palmitoyl- (16:0) and stearoyl-CoA (18:0) to palmitoleoyl- (16:1) and oleoyl-CoA (18:1), respectively. These monounsaturated FA are integral components of phospholipids, TG, wax esters, and cholesterol esters (42). Therefore, the regulation of SCD-1 is crucial in the formation of these lipid fractions and essential for TG synthesis. Decreases in SCD-1 expression or activity have been associated with reduced TG levels in plasma and liver (43–45), as well as increased insulin sensitivity (22,23). These effects also may be due to increased β-oxidation, which occurs with reduced SCD-1 expression (22). Both CLA (11–14) and thiazolidinediones (17,18) have been reported to reduce SCD-1 expression and activity or alter FA composition, specifically decreasing monounsaturated FA, indicating a reduction of SCD-1 activity. In this study, CLA and TZD treatment decreased the SCD-1 indices in neutral and phospholipid fractions in livers of ZDF rats, suggesting a reduction of SCD-1 activity. Although ZDF rats lack responsiveness to leptin, a regulator of SCD-1 (46), CLA and TZD reduced SCD-1 indices, indicating these effects are independent of a leptin:leptin receptor-mediated mechanism. However, perhaps due to the unresponsiveness to leptin, ZDF rats, regardless of diet, had significantly higher SCD-1 indices than lean rats. Conversely, lean rats respond to leptin and had relatively low SCD-1 indices, which were not further reduced by either diet. This suggests that despite the presence of down-regulators of SCD-1, such as leptin, CLA, or TZD, other regulatory mechanisms maintain a basal level of SCD-1 activity. Synthesis of hepatic TG and cholesterol ester are dependent on SCD-1 activity, and therefore maintenance of the activity of this enzyme is crucial to lipid homeostasis (43).

Impairments in insulin sensitivity have been closely linked to increases in plasma FFA and TG concentrations. Studies in which both glucose metabolism and lipid accumulation were measured suggest that a reduction in plasma, as well as tissue, TG correlates with improved glucose tolerance and insulin sensitivity (47). Thiazolidinediones act, in part, by promoting uptake of circulating FFA and storage in the adipose tissue, thus lowering plasma FFA and allowing for improved insulin sensitivity. Previous research showed that CLA reduced plasma TG and FFA levels in both rats and mice (3,4,6–8,48). The exact mechanism by which CLA is able to reduce plasma TG and FFA is largely unknown, but the enhanced uptake of circulating FFA due to increased lipid oxidation has been suggested as a possible mechanism (8,49). The present study shows that CLA decreased plasma FFA and TG in both lean and ZDF rats and to the same degree as TZD (except ZDF-CLA plasma TG). Other studies have reported that CLA improved fasting glucose and hyperinsulinemia in obese or diabetic rats (4–6). Accordingly, we report that CLA normalized glucose tolerance in ZDF rats. Importantly, in this study, CLA was as effective as TZD in improving glucose tolerance, and CLA did not affect glucose tolerance in nonobese, lean rats.

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Olive Oil Consumption and Weight Change: The SUN Prospective Cohort Study

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ABSTRACT: The aim of this dynamic prospective follow-up study was to assess the association between olive oil consumption and the likelihood of weight gain or the incidence of overweight or obesity in a large Mediterranean cohort of 7,368 male and female Spanish university graduates (the SUN Project) who were followed for a median period of 28.5 mon. A validated Food Frequency Questionnaire was administered at baseline, and respondents also completed a follow-up questionnaire after 28.5 mon. Changes in participants' consumption of olive oil and their weight were assessed during follow-up. A higher baseline consumption of olive oil was associated with a lower likelihood of weight gain, although the differences were not statistically significant. The adjusted difference in weight gain (kg) was –0.16 [95% confidence interval (CI): –0.42 to +0.11] for participants in the upper quintile of olive oil consumption (median: 46 g/d) compared with those in the lowest quintile (median: 6 g/d). For participants with a high baseline consumption of olive oil whose olive oil consumption also increased during follow-up, we found a slightly increased but nonsignificant risk of incidence of overweight or obesity (adjusted odds ratio = 1.19, 95% CI: 0.73 to 1.95). Our study, carried out in a sample of free-living people, shows that a high amount of olive oil consumption is not associated with higher weight gain or a significantly higher risk of developing overweight or obesity in the context of the Mediterranean food pattern.

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The role of diet on ischemic heart disease has been studied for almost a century, and substantial evidence about the protection provided by some nutrients and foods is currently available (1,2). One of the most influential seminal works came from the Seven Countries Study (3), which introduced the concept of the cardioprotective properties of the food habits in Mediterranean populations, where olive oil is the main source of fat (1,4). For the last few decades, several population studies have supported its potential cardioprotective effect. The Mediterranean dietary pattern has consistently been associated with a lower mortality from all causes in prospective studies (5–7), and also to a lower incidence of nonfatal coronary events in two case-control studies (8–10). In fact, the U.S. Food and Drug Administration has endorsed olive oil consumption for cardiovascular prevention (11).

However, some criticism (12) has been raised about recommending virgin olive oil consumption to protect from cardiovascular diseases because it is presumed to increase the risk of overweight or obesity. This presumption is based on the nutritional content of olive oil (100% fat). Currently, obesity is one of the most important public health problems (13), and the possibility of increasing population levels of obesity could hinder the promotion of a fat-rich traditional Mediterranean food pattern in which the source of fat is mainly monounsaturated FA (MUFA) provided by olive oil.

Olive oil is an energy-dense food that provides a large amount of fat in the diet. In contrast. carbohydrate-rich diets (i.e., those reducing fat intake) have been associated with substantial weight gain, and low-carbohydrate diets (i.e., those relatively rich in fat) have become increasingly popular. Hence, it is necessary to better assess the role of the high-fat/low-carbohydrate approach in overweight and obesity among free-living subjects (14,15).

The purpose of our study was to address more precisely the question of whether the promotion of healthy lipids such as olive oil would increase the obesity epidemic. Here, we estimate the association between total olive oil consumption and the likelihood of weight gain or of becoming overweight or obese in a longitudinal study with high between-subjects variability conducted in a Mediterranean country, where olive oil is usually consumed. This setting provides a unique opportunity to test this hypothesis following one of the research recommendations for obesity issued by the American Heart Association in its Prevention Conference VII (16).

METHODS

Study population. The SUN Project (Seguimiento Universidad de Navarra, i.e., Follow-up University of Navarra) is a large Spanish longitudinal study (a prospective cohort) that was designed in collaboration with the Harvard School of Public Health using a methodology similar to that used in large U.S. cohorts such as the Nurses' Health Study (17) and the Health Professionals Follow-up Study (18).

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Abbreviations: BMI, body mass index; CI, confidence interval; FFQ, food frequency questionnaire; MET, metabolic equivalent(s); MUFA, monounsaturated fatty acids; OR, odds ratio(s).

The SUN cohort is followed up using biennial mailed questionnaires, with a follow-up rate exceeding 90% for the first 2-yr period (19,20). The recruitment of participants started in December 1999 and is permanently open. All participants had a university degree (at least college) at the onset of the study. The data set of the SUN project incorporated 17,170 participants up to December 2004. Recruitment is ongoing, as this is a dynamic cohort study. Previous analyses of this cohort were published earlier, when the number of recruited participants was smaller (21).

For the present analyses, we included participants who had already been followed up after a median of 28.5 mon (n = 9,000). Participants who reported excessively high or low values for total energy intake (less than 800 kcal/d in men and 600 kcal/d in women or more than 4,200 kcal/d in men and 3,500 kcal/d in women), missing values in variables of interest in the analysis, and pregnant women were excluded, leaving a total of 7,368 participants available for analyses.

The study was approved by the Human Research Ethics Committee at the University of Navarra. Voluntary completion of the first self-administered questionnaire was considered to imply informed consent.

Assessment of dietary and nondietary exposures. Dietary and nondietary exposures were assessed through a self-administered questionnaire (baseline questionnaire) including a 136-item food frequency questionnaire (FFQ) previously validated in Spain (22). This questionnaire also included different questions related to lifestyle, with 46 items for men and 54 items for women. Sociodemographic variables (sex, age, marital status, university degree, and employment) anthropometric data (weight, height, body image, and weight change), health-related habits (e.g., smoking status, alcohol consumption, and physical activity), and medical history information (medication use, cholesterol level, blood pressure, and family history of several diseases) were also collected. The reproducibility and validity of the self-reported weight was assessed in a representative subsample of the cohort (n = 70). The mean relative error in self-reported weight was 1.5%. The correlation coefficient between measured and self-reported weight was 0.991 [95% confidence interval (CI): 0.986–0.994] (23). We inquired about 17 activities to quantify the amount of physical activity, and a metabolic equivalent index (MET-h/wk) was computed (24).

Nutrient scores were calculated as frequency × nutrient composition of a specified portion size, where frequencies were measured in nine categories (6+/d, 4–6/d, 2–3/d, 1/d, 5–6/wk, 2–4/wk, 1/wk, 1–3/mon, and never or almost never for each food item). Nutrient intake scores were computed using an *ad hoc* computer program specifically developed for this aim. A trained dietitian updated the nutrient data bank using the latest available information included in food composition tables for Spain (25,26).

Assessment of variables of interest. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Information on weight was collected at baseline and in the follow-up questionnaire (after a median of 28.5 mon of follow-up). The outcomes at the end of follow-up were: (i) incident overweight or obesity (participants with a baseline BMI value lower than 24.9 kg/m^2 and with a BMI ≥ 25 kg/m^2 after follow-up), and (ii) change in weight after 28.5 mon of follow-up. A careful procedure, combining several items in the FFQ, was used to assess the exposure to olive oil consumption. Baseline total olive oil consumption was calculated from the nutrient score of olive oil consumption in the FFQ, also taking into account the amount and type of olive oil used for cooking or frying and the use of olive oil as a salad dressing or a spread on bread or other foods. Information on changes in olive oil consumption (consistent, increased, or reduced) was collected in the follow-up questionnaire.

Statistical analysis. We estimated the association between quintiles of baseline olive oil consumption (our sample was categorized into 5 groups, from low consumption—first quintile, Q1—to high consumption—last quintile, Q5—and the subsequent change in weight using linear regression models. We considered the lowest quintile (Q1) of total baseline olive oil consumption to be the reference category of exposure and weight change during follow-up as the outcome. Therefore, each regression coefficient can be interpreted as the mean difference in weight gain between the upper categories of olive oil consumption (Q2 to Q5) and the lowest category (Q1).

In addition, logistic regression models were fit to assess the relationship between baseline olive oil consumption as the exposure and the risk of incident overweight or obesity (BMI ≥ 25 kg/m^2) as the outcome. Odds ratios (OR) and their CI were calculated for each of the four upper quintiles considering the lowest quintile of baseline olive oil consumption (Q1) as the reference category. In consequence, the OR represent an estimation of relative risks. They indicate how much more likely it is that someone who is exposed to upper levels of olive oil consumption (Q2 to Q5) develop overweight or obesity (BMI ≥ 25 kg/m^2) as compared with someone who is exposed to the lowest level (Q1). Tests of linear trends across increasing quintiles of intake were calculated by assigning the median intake to each quintile and treating the quintiles in each model as continuous variables.

All dietary intakes were adjusted for total energy intake using the residuals method (27). We fit a crude model (univariate, i.e., without any adjustment), an age- and gender-adjusted model, and a multivariate-adjusted model including the following variables: total energy intake (kcal/d), energy-adjusted fiber intake (g/d), energy-adjusted vegetable consumption (g/d), physical activity during leisure time (MET-h/wk), smoking status (never smoked, smoker, or former smoker), snacking between meals (yes/no), TV viewing (h/wk), and baseline BMI (kg/m^2) (for models using overweight or obesity as the outcome) or baseline weight (for models using weight change). We evaluated all first-order multiplicative interactions (effect modifications) through product terms.

To assess the joint exposure to both baseline total olive oil consumption and changes in olive oil consumption during follow-up (increased vs. consistent or reduced), we considered participants with both low baseline olive oil consumption (lowest tertile) and no increment in consumption (consistent or reduced)

during follow-up as the reference category. We built five other categories combining both exposures, i.e., baseline total olive oil consumption (three categories: low, moderate, and high) and changes in olive oil consumption during follow-up (two categories: consistent or reduced vs. increased). To these combined categories we applied the same statistical procedures previously described for analyzing only baseline consumption as the exposure (linear regression models for weight change and logistic regression models for incident overweight or obesity). All *P* values presented are two-tailed; $P < 0.05$ was considered statistically significant.

RESULTS

Olive oil consumption was higher among women, participants with a lower baseline weight, and current and former smokers. Higher olive oil consumption was associated with a higher vegetable consumption. As expected, participants with higher olive oil consumption exhibited a higher total fat intake ($P < 0.001$) (41% of total energy intake in the fifth quintile) because of increased MUFA intake (20% of total energy in the fifth quintile) (Table 1).

The results for the association between baseline consumption of olive oil and subsequent weight change (as a continuous outcome) were not significant (Table 2). However, we found negative point estimates for the fourth and fifth quintiles of energy-adjusted olive oil consumption that did not change after further adjustment for age and gender or after multivariate adjustment, suggesting that a higher baseline consumption of olive oil was associated with a *lower* likelihood of weight gain, although differences were not statistically significant.

During a median follow-up of 28.5 mon, we observed 405 new (incident) cases of overweight or obesity (BMI ≥ 25 kg/m^2) among 5,356 participants initially free of overweight or obesity. No significant association was observed between olive oil consumption and the risk of developing overweight or obesity during follow-up. The crude results did not substantially change when we used a multivariate logistic regression model to adjust for age, gender, total energy intake, fiber intake, vegetable consumption, leisure-time physical activity, smoking, snacking between meals, TV viewing, and baseline BMI (Table 3).

When we assessed the joint exposure to baseline consumption (tertiles of olive oil) and subsequent changes in consumption during follow-up (dichotomous: increased vs. consistent or reduced), we observed an increase in average weight after follow-up in all categories of olive oil consumption. However, when we compared weight change across categories of olive oil consumption, the highest weight gain was observed precisely for those with the lowest baseline consumption who also did not increase their consumption during follow-up (mean weight gain: +0.85 kg). The lowest weight gain was observed among those in the moderate category of baseline olive oil consumption who increased their consumption during follow-up (+0.42 kg). To control for potential confounding in these comparisons, we used multivariate linear regression modeling (Table 4). We found a significantly lower weight gain among

TABLE 1
Characteristics of Participants According to Quintiles (Q)[a] of Baseline Olive Oil Consumption[b]

	Q1	Q2	Q3	Q4	Q5	P[c]
Participants (*n*)	1,473	1,474	1,474	1,474	1,473	<0.001
Olive oil consumption (g/d)	6 (5)	10 (4)	13 (5)	25 (6)	46 (8)	<0.001
Age (yr)	35 (12)	36 (12)	38 (13)	36 (12)	38 (12)	<0.001
Women (%)	48	55	56	66	67	<0.001
Baseline weight (kg)	68 (14)	67 (13)	67 (14)	66 (13)	67 (13)	<0.001
Baseline BMI (kg/m^2)	23 (3)	23 (3)	24 (4)	23 (3)	23 (4)	0.008
Weight change (kg)	+0.70 (4)	+0.79 (4)	+0.68 (4)	+0.60 (4)	+0.54 (4)	0.428
Physical activity[d] (MET-h/wk)	20 (23)	18 (22)	19 (22)	20 (23)	18 (19)	0.022
TV viewing (h/wk)	11 (6)	11 (6)	10 (6)	10 (6)	10 (6)	<0.001
Current smokers (%)	23	25	25	25	27	0.322
Former smokers (%)	23	23	27	26	30	<0.001
Vegetable consumption (g/d)	517 (476)	488 (309)	475 (336)	593 (403)	605 (558)	<0.001
Fruit consumption (g/d)	389 (452)	324 (283)	297 (263)	381 (329)	355 (310)	<0.001
Total energy intake (kcal/d)	3,013 (1176)	2,376 (712)	2,116 (808)	2,654 (820)	2,689 (1072)	<0.001
Total fat intake (% of energy)	36 (7)	36 (6)	36 (8)	38 (6)	41 (7)	<0.001
MUFA (% of energy)	14 (3)	15 (3)	16 (5)	17 (3)	20 (4)	<0.001
SFA (% of energy)	13 (4)	13 (3)	13 (4)	12 (3)	12 (3)	<0.001
PUFA (% of energy)	6 (2)	5 (2)	5 (2)	5 (2)	6 (2)	<0.001
Carbohydrate intake (% of energy)	45 (8)	44 (8)	43 (8)	43 (7)	41 (7)	<0.001
Protein intake (% of energy)	18 (3)	19 (4)	19 (4)	18 (3)	17 (3)	<0.001
Fiber intake (g/d)	32 (22)	27 (12)	25 (13)	31 (16)	30 (19)	<0.001
Snacking between meals (%)	36	35	32	36	37	0.032

[a]Q1–Q5: Lowest to highest quintile of olive oil consumption.
[b]Data are expressed as means (SD) unless otherwise indicated.
[c]*P* value was tested by Pearson's chi square for categorical variables and ANOVA for continuous variables.
[d]Physical activity was computed as metabolic equivalents (MET)-hours per week using the duration per week of various (17) forms of exercise. BMI, body mass index; MUFA, monounsaturated FA; SFA, saturated FA.

TABLE 2
Estimates [regression coefficients and 95% confidence intervals (CI)] for the Association Between Energy-Adjusted Quintiles (Q)[a] of Baseline Olive Oil Consumption and the Subsequent Change in Weight (kg)

	Q1	Q2	Q3	Q4	Q5
Participants (*n*)	1473	1474	1474	1474	1473
Weight change [kg, (95% CI)]	+0.70 (+0.51 to +0.90)	+0.79 (+0.61 to +0.97)	+0.68 (+0.49 to +0.87)	+0.60 (+0.49 to +0.87)	+0.54 (+0.35 to +0.74)
Differences in weight change with respect to Q1					
Crude (regression coefficient, β)	0 (ref.)	+0.09 (−0.18 to +0.35)	−0.02 (−0.23 to +0.25)	−0.10 (−0.36 to +0.17)	−0.16 (−0.42 to +0.11)
Model 1[b] (regression coefficient, β)	0 (ref.)	+0.09 (−0.18 to +0.36)	+0.02 (−0.25 to +0.29)	−0.10 (−0.37 to +0.17)	−0.14 (−0.41 to +0.13)
Multivariate adjusted model[c] (regression coefficient, β)	0 (ref.)	+0.09 (−0.18 to +0.35)	+0.04 (−0.23 to +0.30)	−0.10 (−0.37 to +0.17)	−0.16 (−0.43 to +0.11)

[a]Q1–Q5: Lowest to highest quintile of olive oil consumption.
[b]Adjusted for gender, age (yr), and a quadratic term for age to account for departure from linearity.
[c]Additional adjustment for total energy intake (kcal/d), energy-adjusted fiber intake (g/d), energy-adjusted vegetable consumption (g/d), leisure-time physical activity (MET-h/wk), smoking status (never smoked, smoker, or former smoker), snacking between meals (yes/no), TV viewing (h/wk), and baseline weight (kg). An interaction term (age·baseline weight, $P < 0.001$) was also added.

TABLE 3
Odds Ratios (OR) (95% CI) of Incident Overweight or Obesity According to Energy-Adjusted Quintiles (Q)[a] of Baseline Olive Oil Consumption in 5,356 Participants of the SUN Study

	Q1	Q2	Q3	Q4	Q5	*P* for trend
Participants (*n*)	1071	1071	1072	1071	1071	
No. of cases	77	84	85	79	80	
Crude OR	1 (ref.)	1.10 (0.98 to 1.52)	1.11 (0.81 to 1.53)	1.03 (0.74 to 1.42)	1.04 (0.75 to 1.44)	0.95
Age- and gender-adjusted OR	1 (ref.)	1.17 (0.85 to 1.63)	1.20 (0.86 to 1.66)	1.28 (0.92 to 1.79)	1.25 (0.89 to 1.74)	0.23
Multivariate adjusted[b] OR	1 (ref.)	0.97 (0.67 to 1.40)	0.95 (0.65 to 1.40)	1.05 (0.72 to 1.53)	1.11 (0.76 to 1.61)	0.45

[a]Q1–Q5: Lowest to highest quintile of olive oil consumption.
[b]Additional adjustment for total energy intake (kcal/d), energy-adjusted fiber intake (g/d), energy-adjusted vegetable consumption (g/d), leisure-time physical activity (MET-h/wk), smoking status (never smoked, smoker, or former smoker), snacking between meals (yes/no), TV viewing (h/wk), baseline BMI (kg/m^2). For other abbreviations see Tables 1 and 2.

TABLE 4
Regression Coefficients (95% CI) for the Association of Weight Change (kg) with the Joint Exposure to Changes in Olive Oil Consumption (reduced or consistent vs. increased) During Follow-up and Energy-Adjusted Baseline Olive Oil Consumption (tertiles, T[a])

	Tertiles of energy-adjusted baseline olive oil consumption					
	Low baseline consumption (T1)		Moderate baseline consumption (T2)		High baseline consumption (T3)	
Change in olive oil consumption	Consistent or reduced	Increased	Consistent or reduced	Increased	Consistent or reduced	Increased
Participants (*n*)	1876	580	1935	521	1964	492
Weight change [kg, (95% CI)]	+0.85 (+0.69 to 1.01)	+0.47 (+0.16 to +0.79)	+0.67 (+0.50 to +0.84)	+0.42 (+0.13 to +0.71)	+0.57 (+0.41 to +0.74)	+0.78 (+0.45 to +1.08)
Differences in weight change with respect to T1 and consistent or reduced consumption during follow-up						
Crude (regression coefficient, β)	0 (ref.)	−0.38 (−0.72 to −0.03)	−0.18 (−0.42 to +0.05)	−0.43 (−0.79 to −0.07)	−0.28 (−0.51 to −0.05)	−0.07 (−0.43 to +0.30)
Model 1[b] (regression coefficient, β)	0 (ref.)	−0.36 (−0.70 to −0.02)	−0.16 (−0.39 to +0.08)	−0.37 (−0.73 to −0.02)	−0.28 (−0.51 to −0.04)	−0.05 (−0.42 to +0.32)
Multivariate adjusted model[c] (regression coefficient, β)	0 (ref.)	−0.38 (−0.72 to −0.04)	−0.16 (−0.39 to +0.08)	−0.37 (−0.73 to −0.01)	−0.30 (−0.53 to −0.06)	−0.07 (−0.43 to +0.30)

[a]T1–T3: Lowest to highest tertile of olive oil consumption.
[b]Adjusted for gender, age (yr), and a quadratic term for age to account for departure from linearity.
[c]Additional adjustment for total energy intake (kcal/d), energy-adjusted fiber intake (g/d), energy-adjusted vegetable consumption (g/d), leisure-time physical activity (MET-h/wk), smoking status (never smoked, smoker, or former smoker), snacking between meals (yes/no), TV viewing (h/wk), and baseline weight (kg). An interaction term (age·baseline weight, $P < 0.001$) was also added. For other abbreviation see Table 2.

TABLE 5
Odds Ratios (95% CI) of Incident Overweight or Obesity According to the Joint Exposure to Energy-Adjusted Tertiles (T)[a] of Baseline Olive Oil Consumption and Change in Olive Oil Consumption During Follow-up (reduced or consistent vs. increased) in 5,356 Participants of the SUN Study

Change in olive oil consumption	Tertiles of energy-adjusted baseline olive oil consumption					
	Low baseline consumption (T1)		Moderate baseline consumption (T2)		High baseline consumption (T3)	
	Consistent or reduced	Increased	Consistent or reduced	Increased	Consistent or reduced	Increased
Participants (*n*)	1370	425	1387	341	1493	340
No. new cases	102	28	103	33	110	29
Crude OR	1 (ref.)	0.88 (0.57–1.35)	1.00 (0.75–1.33)	1.33 (0.88–2.01)	0.99 (0.75–1.31)	1.16 (0.75–1.78)
Age- and gender-adjusted OR	1 (ref.)	0.89 (0.57–1.38)	1.09 (0.81–1.46)	1.30 (0.85–1.98)	1.22 (0.91–1.62)	1.38 (0.89–2.14)
Multivariate adjusted[b] OR	1 (ref.)	0.82 (0.51–1.33)	0.94 (0.68–1.31)	1.28 (0.80–2.05)	1.12 (0.81–1.55)	1.19 (0.73–1.95)

[a]T1–T3: Lowest to highest tertile of olive oil consumption.
[b]Additional adjustment for total energy intake (kcal/d), energy-adjusted fiber intake (g/d), energy-adjusted vegetable consumption (g/d), leisure-time physical activity (MET-h/wk), smoking status (never smoked, smoker, or former smoker), snacking between meals (yes/no), TV viewing (h/wk), and baseline BMI (kg/m^2). For abbreviations see Tables 1–3.

participants with moderate baseline consumption who increased their consumption during follow-up than among participants in the reference category (low baseline + no increment). The adjusted difference in weight gain was −0.37 kg (95% CI: −0.73 to −0.01). Similarly, participants with high baseline olive oil consumption and no increment in consumption during follow-up experienced a statistically significant lower weight gain than participants in the reference category (adjusted difference = −0.30 kg; 95% CI: −0.53 to −0.06). No significant differences in weight gain were found between those with the highest baseline consumption who also increased their consumption and the reference category (Table 4). Moreover, the Pearson correlation coefficient between baseline olive oil consumption and subsequent body weight change (both as continuous variables) was −0.020 ($P = 0.081$).

In Table 5 we show the results for the risk of becoming overweight or obese when we restricted our analyses only to those participants initially free of overweight or obesity ($n = 5,356$). We found no significant differences in the risk of developing overweight or obesity for the categories built according to the joint exposure to baseline consumption and changes in consumption of olive oil compared with those in the reference category (both baseline consumption and consistent or reduced change during follow-up). For those with the highest baseline consumption who also increased their consumption during follow-up, the OR was 1.19 (95% CI: 0.73 to 1.95). When we repeated all these analyses (both linear and logistic regression modeling), excluding those participants with prevalent cancer or cardiovascular disease, we obtained very similar results (data not shown).

We used energy-adjusted olive oil consumption (27) throughout all the previous analyses. However, we also repeated all the analyses without adjusting for total energy intake, and the only noticeable change was observed among those who had high baseline olive oil consumption and who also increased their consumption during follow-up. Their adjusted OR for overweight or obesity changed from 1.19 (95% CI: 0.73 to 1.95) to 1.33 (95% CI: 0.69 to 2.56).

DISCUSSION

In this 28.5-mon follow-up study of a large, free-living Mediterranean cohort with high between-subjects variability in olive oil consumption, we did not find a significant positive association between olive oil consumption and weight gain or between olive oil intake and the risk of overweight or obesity. In fact, most point estimates for weight change suggested an inverse association, i.e., lower weight gain for those with higher olive oil consumption, and therefore with a higher proportion of their total energy coming from lipids (mainly from MUFA).

In spite of the high caloric density represented by olive oil consumption, our results could be partially explained by different FA displaying differential effects on weight gain through differences in sensitivity to neurotransmitters, to the intestinal peptide enterostatin, or to specific influences of FA on thermogenesis (28,29). Additionally, between-nutrient interactions may be important, because olive oil in Mediterranean countries is consumed within a bread-rich dietary pattern, where it is commonly used for dressing legumes, vegetables, and salads. In this context, a possible interaction between starch and lipids in the upper part of the small intestine after fat intake, which may slow the rate of starch digestion and reduce the glycemic response, has been reported (30). Other reasons to support our results are based on the fact that, as the dietary fat load is increased, proportionate increases occur in fecal fat losses (31). Moreover, high levels of olive oil consumed might not be completely absorbed and digested (32).

The promotion of a Mediterranean diet with a high percentage of fat from olive oil consumption to protect from cardiovascular diseases presents some unclarified questions for public health. A major reason for concern is that the prevalence of obesity in the Mediterranean population is one of the highest in Europe. In consequence, some authors have viewed the recommendation of following an olive oil-rich Mediterranean diet as one of the major causes responsible for this fact (12). However, this view is not based on reliable epidemiological evidence, and, to our knowledge, this is the first *prospective* large-sample

study that has specifically assessed the role of olive oil on the risk of weight gain in a Mediterranean country. The confirmation of a lack of weight gain among our participants exposed to high levels of olive oil consumption should allay fears that the promotion of lipid-rich Mediterranean food patterns may lead to increased overweight or obesity in the population.

Additionally, our results demonstrated that although participants who consumed high amounts of olive oil (upper quintile) had a very high total fat intake (around 41% of total calories), their percentage of total calories derived from saturated fats was lower, whereas their consumption of vegetables was higher.

Our data are consistent with four cross-sectional studies in which no relationship or a very weak association was observed between olive oil consumption and obesity or weight gain (33–36). Recently, Trichopoulou *et al*. (36) showed that adherence to a Mediterranean diet score (including the ratio of monounsaturated to saturated lipids) was essentially unrelated to BMI in a large Greek cohort. However, none of these previous studies used a prospective design and their analyses were only cross-sectional, having the inherent threat of a reverse causation bias. Thus, if subjects who watch themselves gaining weight tend to restrain from consuming olive oil because they believe in a weight-gaining effect of olive oil, any cross-sectional analysis will find an *inverse* association, but this association would be the result of a bias. Our study is less susceptible to this bias because of the longitudinal prospective design we followed.

The results from this study confirm, with a greater number of individuals and with a specific focus on olive oil consumption rather than on the Mediterranean dietary pattern, the results previously obtained in our cohort (37) by addressing not only weight gain but also the risk of developing overweight or obesity.

A potential limitation in our study is that most of the results we report here are not statistically significant, thus raising the issue that perhaps this study might have been unable to capture a positive or negative relationship owing to low statistical power and a type 2 error. The statistical power of our dichotomous analyses (overweight or obesity as the outcome) for a relative risk ≥2.00 is higher than 95% (the power would be 53% if the relative risk were 1.5, and we would have insufficient statistical power only for relative risks lower than 1.5). However, the statistical power approached 100% when we treated weight changes as continuous variables to apply linear regression modeling. It is precisely in this highly powered analysis, using a continuous variable (weight change) as the outcome, that we found results suggesting, in any case, that higher olive oil consumption was related to *lower* weight gain.

Because of the observational nature of our study, we cannot exclude residual confounding because it could theoretically affect the observed lack of association. We controlled for potential confounding by taking into account most known risk factors for obesity that are plausibly also associated with olive oil consumption. We also acknowledged that our estimates of total energy intake or of the consumption of some foods, as measured by the FFQ, may present some degree of measurement error, which is to be expected in nutritional epidemiology (22,38); therefore, the variance in total energy intake may be seen as large. This large variability is usual in most studies of nutritional epidemiology (39,40); however, it might limit our ability to capture a positive or negative relationship. In addition, a further potential limitation of our study is the reliance on self-reported body weight. Nevertheless, a substudy in our cohort showed that self-reported weight can be considered valid among highly educated participants (23), as other similar cohorts have also shown (41). Our cohort is nonrepresentative of the general population because all participants have attained a high educational level. However, this potential limitation to generalization of the results is not a sufficiently strong reason to limit the validity of our findings, because it is implausible to think that the effects of olive oil on weight gain depend on participants' educational level (42).

Therefore, with these results we can provide evidence that the risk of obesity is not increased in subjects who follow an olive oil-rich Mediterranean food pattern. The belief that olive oil consumption increases the risk of obesity in Mediterranean populations is not supported by any reliable evidence. However, epidemiological evidence supports the major role of a sedentary lifestyle as a more likely explanation for the increasing obesity epidemic in Mediterranean countries (43,44).

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Effects of Soy or Milk Protein During a High-Fat Feeding Challenge on Oxidative Stress, Inflammation, and Lipids in Healthy Men

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ABSTRACT: Soy isoflavones may impede atherogenic processes associated with cardiovascular disease. Research suggests that the postprandial generation of TG-rich remnants contributes to the development of atherosclerosis. The purpose of the current study was to determine if 39 g soy (85 mg aglycone isoflavones, treatment) compared with 40 g milk protein (0 mg aglycone isoflavones, control) in combination with a high-fat meal can modify postprandial, atherogenic-associated events and biomarkers for oxidative stress, inflammation, and thrombosis. Fifteen healthy men (20–47 yr) participated in a double-blind cross-over meal-challenge study occurring on two nonconsecutive days. The study meals consisted of two high-fat apple muffins consumed with either a soy or milk shake (229 mL, 41% fat, 41% carbohydrate, and 18% protein). Blood samples were obtained at baseline (fasted) and hours two, four, and six postprandial. Plasma TG significantly increased in both treatment and control meal challenges compared with baseline. There were no significant differences ($P > 0.05$) between treatment (soy) and control (milk) for *ex vivo* copper-induced LDL oxidation, serum C-reactive protein, serum interleukin-6 (IL-6), serum fibrinogen, or plasma lipids (total cholesterol, HDL, LDL, TG). IL-6-concentrations significantly decreased as a function of time during either meal challenge ($P = 0.005$). These data suggest that consumption of soy or milk protein in conjunction with a high-fat meal does not acutely modify postprandial oxidative stress, inflammation, or plasma lipid concentrations in young, healthy men.

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Cardiovascular disease (CVD) has been the leading cause of death in the United States for almost a century. Nearly 2,600 Americans die of CVD each day, an average of 1 death every 34 s (1). More than 20 years ago, Zilversmit (2) suggested that the postprandial generation of TG-rich remnants contributed to the development of atherosclerosis. Investigators have proposed that Western populations exist in a predominantly chronic postprandial state due to frequent meal consumption throughout the day (3) and this lengthened hyperlipidemic state may contribute to prolonged vascular injury and the initiation and development of atherosclerosis (3). LDL oxidation increases risk of atherosclerosis (4,5), and investigators suggest that postprandial LDL are more easily oxidized than fasting LDL (6). Reactive oxygen species produced in the vessel wall in response to initial endothelial injury can also mediate adverse downstream cellular responses associated with atherosclerosis and CVD, such as inflammation, platelet aggregation, thrombosis, and DNA damage (7–10).

High-fat meals have been shown to increase glucose, insulin (11), and TG concentrations (11,12), impair (13–18) or have minimal effect (19) on endothelial function; increase postprandial lipid hydroperoxides (biomarkers of oxidative stress) (20,21); and reduce the time needed to oxidize LDL particles (22). Additionally, meals high in fat have been shown to increase pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) plasma concentrations (23) and either increase (24) or have no effect (16) on C-reactive protein (CRP), a biomarker of systemic inflammation and CVD risk (25–27). Fibrinogen and factor VII (VII-c) play a role in atherosclerotic plaque development (28) and are CVD hemostatic risk biomarkers (29). Studies report postprandial-induced activation of plasma factor VII-c (30) but not fibrinogen concentrations (30,31) following a high-fat meal. Collectively, these data encourage investigators to explore the health benefits of consuming specific foods or nutrients that decrease meal-induced oxidative stress and inflammatory and hemostatic processes.

Consumption of soy-containing foods may, in part, explain the low incidence of CVD and hormone-dependent cancers in the Asian population (32). Western consumption of soy products has surged since 1999 when the FDA released a health claim for the cholesterol-lowering potential of soy products (33,34). Genistein and daidzein are the predominant isoflavones in soy foods and have been shown to protect against LDL oxidation by reducing formation of preformed lipid hydroperoxides (35), decreasing tyrosine nitration (36,37), and increasing lipoprotein particle antioxidant potential owing to their lipophilic phytoestrogen structures (38–42).

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Abbreviations: AUC, area under the curve; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; HI, high isoflavone; IL-6, interleukin-6; LI, low isoflavone; NRL, Nutrition Research Laboratory; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; T0, time point hour 0, pre-meal challenge; T2, T4, T6, time point hours 2, 4, 6 post-meal challenge, respectively; TC, total cholesterol; TNF-α, tumor necrosis factor-α.

Although these CVD-protective mechanisms are promising, intervention studies with soy protein and isoflavones lasting 1–2 mon have yielded conflicting results. For instance, some studies report a reduction in the susceptibility of LDL to oxidative stress (42–44), whereas others show no effect on *in vivo* or *ex vivo* oxidative stress markers (45–47). Genistein is a potent inhibitor of tyrosine kinase activity (48), and platelets possess several protein-tyrosine kinase domains that undergo phosphorylation when activated. Results from *in vitro* studies show genistein is able to block thrombin-induced human platelet aggregation by inhibiting tyrosine kinase activity (49,50) and there is considerable interest in the ability of soy isoflavone phytopharmaceuticals potentially to modulate IL-6 gene expression and activity (51). Very few studies have examined the effect of soy and/or isoflavones on inflammatory (IL-6, CRP, TNF-α) or coagulation (fibrinogen) biomarkers (52–55), and none are postprandial (6-h post-meal) studies. For instance, Jenkins and co-workers (54) reported an increase in IL-6 concentration following a 1-mon soy intervention; other investigations showed no effects of diets high in tofu (52) or isoflavones (53,55) on fibrinogen (52,53,55) or factor VII-c concentrations (53,55). On the other hand, isolated soy protein and isoflavones have favorable effects on endothelial vasoactivity and function, as assessed with brachial artery reactivity measurements (56–58).

To date, the postprandial effects of soy consumed with a meal inducing oxidative stress (e.g., high fat) have not been determined. Therefore, the purpose of this study was to determine if 39 g soy protein (85 mg isoflavones, treatment) consumed in combination with a high-fat meal decreased postprandial oxidative stress, inflammation, and hemostatic risk factors compared with a high-fat meal supplemented with 40 g milk protein (0 mg isoflavones, control).

SUBJECTS AND METHODS

Study protocol. Fifteen healthy, nonvegetarian men 20–47 yr of age were recruited to participate in a double-blind cross-over study. On average (mean ± SD), the subjects were 28 ± 9 yr, 180 ± 25 cm tall, 83 ± 10 kg (pre-milk treatment), and 83 ± 11 kg (pre-soy treatment). Exclusion criteria included: (i) regular use (>3 times per week) of a vitamin or mineral supplement in the previous 3 mon; (ii) regular consumption of soy protein (≥2 servings of 6.25 g/d); (iii) known existence of metabolic disorders (thyroid disease, diabetes, liver or renal disease); (iv) use of oral prescription medications (including any antibiotic use within the previous 3 mon) (12); (v) obesity [body mass index (BMI) > 30 kg/m^2); (vi) cigarette smoking within the last 3 yr (59); (vii) known food allergies (soy protein, milk protein, peanuts); (viii) a strict vegetarian status (60); (ix) regular alcohol consumption (>2 drinks/d; 1 drink = 12 oz beer, 6 oz wine, or 1.5 oz distilled alcohol); or (x) regular use of vitamin or herbal supplement (>3 times/wk) with antioxidant capacity.

Participants signed a Human Subjects Informed Consent Form approved by the Montana State University Internal Review Board prior to additional screening and participation in the study. Eligible subjects reported to the NRL at 0700 on two separate, nonconsecutive days in a fasted state (no food or beverage, with the exception of water, for 10 h), and had refrained from any strenuous physical activity or alcohol consumption for at least 24 h prior to the study date. Weight and height were recorded and a fasted blood sample (prechallenge meal; T0) was drawn *via* venipuncture. Participants were given 20 min to consume the high-fat challenge meal. Blood was subsequently drawn at hours 2 (T2), 4 (T3), and 6 (T6) postprandially. Participants remained on-site for the entire 6 h.

The study meal consumed following the baseline blood draw included two high-fat apple crumb muffins made in the Food Science Laboratory on the campus of Montana State University, and either a soy (treatment) or milk (control) protein shake. The soy beverage was made with SUPRO®SOY (Solae™, St. Louis, MO) isolated soy protein, water-washed to retain the naturally occurring isoflavones. The soy shake provided 39.0 g of soy protein (85 mg aglycone isoflavones). The placebo beverage was made with milk protein isolate and provided 40 g of casein/whey combination (0 mg aglycone isoflavones). A banana (100 g) was added to both shakes. The nutrient composition (Nutritionist Pro™ Version 1.2; First DataBank Inc., San Bruno, CA) of the two apple crumb muffins was: 130 mJ, 42.0 g total fat (68.2% of total calories), 26.2 g saturated fat (66.3% of total fat), 6.7 g polyunsaturated fat, 40.6 g carbohydrates (29.2% of total calories), 3.6 g protein (2.6% of total calories), and 33.8 mg cholesterol. Subjects were allowed water *ad libitum* throughout the day. The challenge meal, consisting of two muffins and a soy or milk shake with a banana, was 41% fat, 41% carbohydrate, and 18% protein (Table 1).

Participants were given instructions on how to weigh and record all food and beverages (excluding water) consumed on the 3 d prior to the study date. Diet records were analyzed using the Nutritionist Pro™ Version 1.2 software package. Additionally participants completed a 3-d activity log for the 3 d just

TABLE 1
Challenge Meal Nutrient Composition[a]

Nutrient	Challenge meal with soy protein shake	Challenge meal with milk protein shake
Energy intake (Mj)	229	229
Carbohydrate (g)	100	100
Total protein (g)	45	45
Milk protein (g)	0	40
Soy protein (g)	39	0
Soy isoflavones (mg)	85	0
Total fat (g)	44	44
Saturated fat (g)	26	26
Polyunsaturated fat (g)	7	7
Monounsaturated fat (g)	7	7
P/S ratio[b]	0.25	0.25
Cholesterol (mg)	39	39
Sodium (mg)	895	895
Dietary fiber (g)	4	4

[a]Challenge meal consisted of milk and soy protein shake and two apple-crumb muffins.
[b]Ratio of polyunsaturated FA to saturated FA.

prior to each study date. The Bouchard Three Day Physical Activity Record (61) was used to approximate mean energy expenditure and to assess compliance with the study protocol. The procedure for recording the data was thoroughly explained to the subjects, and a list of activities along with their corresponding ratings was provided.

Assessment of oxidative stress. Isolation of LDL occurred through a sequential density-gradient ultracentrifugation in an Optima TLX Ultracentrifuge (Beckman Instruments, Palo Alto, CA) (62). A 0.5 mL volume of plasma and 0.5 mL of 0.9% sodium chloride solution were centrifuged for 2.5 h (435,680 × *g*; 16°C). The VLDL layer was discarded. A volume of 0.5 mL of 16.7% sodium chloride was added to the remaining HDL and LDL and spun for 2.5 h (435,680 × *g*; 16°C) (63). The isolated LDL was desalted using prepackaged columns (Econo-Pac 10 DG; Bio-Rad, Richmond, CA) filled with bio-Gel P6 desalting gel and subsequently collected and analyzed for protein concentration (63,64). The protein concentration of the LDL eluate was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) adapted to a 96-well microplate (65,66). All standards and samples were analyzed in duplicate at 562 nm (μQuant Universal Microplate Spectrophotometer; Bio-Tek Instruments, Winooski, VT). Isolated and desalted LDL samples were diluted with PBS to achieve a final concentration of 0.10 mg protein/mL (67). One hundred microliter (100 μL) volumes of samples (isolated, desalted, and adjusted for protein concentration) and of blanks (PBS) were incubated in separate wells of a 96-well microplate (Costar; Corning Incorporated, Corning, NY) with 10-μL volumes of 500 mM cupric chloride solution; and the absorbance was read every 10 min for 8 h at 234 nm at 37°C (μQuant Universal Microplate Spectrophotometer). Samples were analyzed in duplicate. Lag time was calculated with an Excel (Microsoft Corporation, Redmond, WA) spreadsheet. The point of intersection of a best-fit line drawn tangent to the propagation phase was defined as the lag time or duration of the lag phase. An increase in lag time is considered beneficial owing to the increased resistance of the LDL to oxidation. The mean of the lag times for duplicate sample wells was used for statistical analysis. The inter- and intra-assay CV were 4 and 2%, respectively.

Assessment of inflammatory and hemostatic risk biomarkers. To determine CRP serum concentrations, frozen (−80°C) blood samples from T0, T2, T4, and T6 for each treatment were thawed. CRP concentrations were determined *via* a nephelometric method using a BN ProSpec (Dade-Behring, Deerfield, IL; instrument located at Bozeman Deaconess Laboratory, Bozeman, MT). The sensitivity of the CRP assay was 0.0175 mg/dL with a range of 0–0.3 mg/dL for a healthy adult.

Blood samples for IL-6 were collected at T0, T2, T4, and T6 for each diet treatment into a serum separator (Becton-Dickson) and allowed to clot for 30 min. Samples were then centrifuged for 10 min at approximately 1000 × *g*, with subsequent storage at −80°C until analysis. Thawed samples were analyzed *via* a Quantikine® Human IL-6α Immunoassay diagnostic kit (R&D Systems Inc., Minneapolis, MN). IL-6 standards and sample concentrations were measured at 450 nm (Model ELIU808IU microplate reader; BioTek). The inter- and intra-assay CV were 4.2 and 7.5%, respectively. The range for the minimum detectable dose for IL-6 with the Quantikine® ELISA was 0.016–0.110 pg/mL (mean = 0.039 pg/mL). Fibrinogen was analyzed in duplicate (bovine fibrinogen reagent hemoalliance reagent) for T0, T2, T4, and T6 for each diet treatment. Blood samples were collected into citrate (0.129 mol/L of citrate/citric acid, 1:10 total volume) and immediately analyzed on an ACL9000 Coagulation Analyzer (Beckman Coulter, Fullerton, CA; instrument located at Bozeman Deaconess Laboratory, Bozeman, MT). .

Assessment of plasma lipid concentrations. Plasma total cholesterol (TC), HDL, and TG concentrations were determined using the Dimension® Automated Chemistry Analyzer (Dade-Behring) (Bozeman Deaconess Hospital). The Friedewald equation (68) was used to calculate the plasma LDL concentration. All samples were run in one continuous batch. Interassay CV for the "low" and "high" LDL control samples were 3.53 and 2.62%, respectively.

Statistical analysis. Power calculations from a pilot project designed to assess the effects of soy protein on postprandial oxidative stress indicated future studies of the effect of soy protein consumption on oxidative stress should use no less than 15 subjects ($\alpha = 0.05$; $\beta = 0.80$). A two-way ANOVA with repeated measures was used to assess treatment (soy vs. milk) differences (lag time, IL-6, CRP, fibrinogen, and blood lipids) for the four time points analyzed using the SPSS (Chicago, IL) statistical program. Differences in baseline values among the time points were assessed with a Bonferroni multiple comparison test. A paired *t*-test was used to determine significance between the means of weight, BMI, energy expenditure, energy intake, and selected macro- and micronutrients between the two treatment types (soy vs. milk). The α level was set at 0.05. Data for all lipid (TC, HDL, LDL, TG) and inflammatory (fibrinogen, IL-6, CRP) variables were analyzed with the incremental and net area under the curve (AUC) (69) methods to assess treatment effects during the postprandial period (data not shown).

RESULTS

Subjects. The following analyses include 15 subjects with complete data for both treatment types. No subjects dropped or withdrew from the study. No significant differences were found for weight between the two study days. There were no significant differences in the nutrient composition of the challenge meals, except that, by design, the treatment meal (soy) was significantly higher in the soy protein compared with the control meal (milk), and whey/casein protein was significantly higher in the control meal vs. treatment meal (Table 1). Energy intake and expenditure, carbohydrate, protein, total fat and selected micronutrients for the 3 d prior to the testing days were not significantly different; however, a significant difference was found for saturated FA intake ($P = 0.04$) (Table 2).

Blood lipid assessment. No significant treatment effect was found for plasma lipid concentrations (TC, HDL, LDL, and

TABLE 2
Energy Intake and Expenditure for 3 d Prior to Study Days[a]

Measure	Soy protein treatment	Milk protein treatment	*P*-Values
Energy expenditure[b] (mJ)	16 ± 3	16 ± 4	0.31
Dietary intake[c]			
Energy intake (mJ)	604 ± 125	642 ± 151	0.24
Total fat (g)	88.7 ± 18.0	103.0 ± 30.2	0.08
Saturated fat (g)	30.9 ± 7.7	36.1 ± 11.0	0.04
Polyunsaturated fat (g)	12.5 ± 6.5	17.5 ± 11.0	0.10
Monounsaturated fat (g)	25.2 ± 9.2	28.8 ± 12.6	0.33
Carbohydrate (g)	340.2 ± 93.2	357.0 ± 80.3	0.26
Total protein (g)	94.8 ± 22.6	86.8 ± 26.4	0.22
Dietary fiber (g)	20.8 ± 7.3	20.3 ± 8.1	0.54
Vitamin C (mg)	106.0 ± 83.5	114.1 ± 95.5	0.65
Vitamin E, α-tocopherol[d] (mg)	24.7 ± 24.4	22.7 ± 23.0	0.54

[a]Values are means ± SD.
[b]Mean value for energy expenditure is reported as assessed by the Bouchard Three Day Physical Activity Record (61).
[c]Dietary intake was assessed using 3-d weighed diet records collected prior to each treatment.
[d]IU × 1.49 = mg.

TABLE 3
Serum Lipid Concentrations (mmol/L) for Each Treatment: Soy or Milk Protein[a,b]

Measure	Treatment	T0	T2	T4	T6	*P*-Values[c]
Cholesterol, total[d]	Milk	3.76 ± 0.72	3.81 ± 0.73	3.84 ± 0.73	3.98 ± 0.74	0.73
	Soy	3.84 ± 0.77	3.91 ± 0.85	3.87 ± 0.80	3.93 ± 0.80	
LDL-cholesterol[e]	Milk	2.21 ± 0.60	2.06 ± 0.56	2.12 ± 0.58	2.30 ± 0.61	0.49
	Soy	2.41 ± 0.70	2.22 ± 0.63	2.13 ± 0.61	2.24 ± 0.63	
HDL-cholesterol	Milk	1.15 ± 0.20	1.16 ± 0.22	1.16 ± 0.19	1.20 ± 0.20	0.47
	Soy	1.12 ± 0.21	1.16 ± 0.23	1.14 ± 0.28	1.16 ± 0.22	
TG[f]	Milk	0.88 ± 0.41	1.27 ± 0.36	1.21 ± 0.50	1.04 ± 0.47	0.61
	Soy	0.90 ± 0.31	1.15 ± 0.47	1.34 ± 0.64	1.15 ± 0.54	

[a]Values are means ± SD.
[b]Values reported at hours T0, T2, T4, and T6.
[c]*P*-values reported for treatment effect.
[d]Significant time effect: T0 < T6, $P = 0.026$.
[e]Significant time effect: T2 < T0, $P = 0.041$.
[f]Signficant time effect: T0 < T2, $P = 0.007$; T0 < T4, $P = 0.001$.

TG) assessed at T0, T2, T4, and T6 (Table 3). However, a significant overall time effect was apparent for plasma TC concentrations ($P = 0.034$) and plasma TG ($P < 0.0001$). A significant decrease in plasma LDL concentration ($P = 0.007$) was observed. Plasma HDL concentration did not change significantly over time ($P = 0.067$). Plasma TC, HDL, LDL, and TG concentrations analyzed as incremental and net AUC were not significantly different (soy vs. milk) (data not shown).

Inflammation and hemostatic risk assessment. There were no significant differences in treatment effect for serum IL-6, CRP, or fibrinogen concentrations (Table 4). IL-6 concentrations significantly decreased as a function of time during either meal challenge ($P = 0.005$) (Table 4). Serum inflammatory variables analyzed as incremental and net AUC were not significantly different (soy vs. milk) (data not shown).

Oxidative stress assessment. Results showed no significant

TABLE 4
Serum Fibrinogen, C-Reactive Protein, and Interleukin-6 Concentrations for Each Treatment, Soy or Milk Protein[a,b]

Measure	Treatment	T0	T2	T4	T6	*P*-Values[c]
Fibrinogen	Milk	252 ± 57	250 ± 51	258 ± 51	261 ± 55	0.19
(mg/dL)	Soy	249 ± 51	264 ± 46	259 ± 46	258 ± 49	
CRP	Milk	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.03	0.09 ± 0.03	0.56
(mg/dL)	Soy	0.07 ± 0.03	0.08 ± 0.03	0.08 ± 0.03	0.07 ± 0.03	
IL-6[d]	Milk	1.6 ± 0.3	1.4 ± 0.36	1.3 ± 0.3	1.3 ± 0.2	0.63
(pg/mL)	Soy	1.6 ± 0.3	1.1 ± 0.1	1.3 ± 0.2	1.5 ± 0.2	

[a]Values are means ± SEM, with Bonferroni adjustment for multiple comparisons.
[b]Values reported at T0, T2, T4, and T6.
[c]*P*-values reported for treatment effect.
[d]Significant time effect, both treatments: T0 > T2, T4. $P = 0.005$.

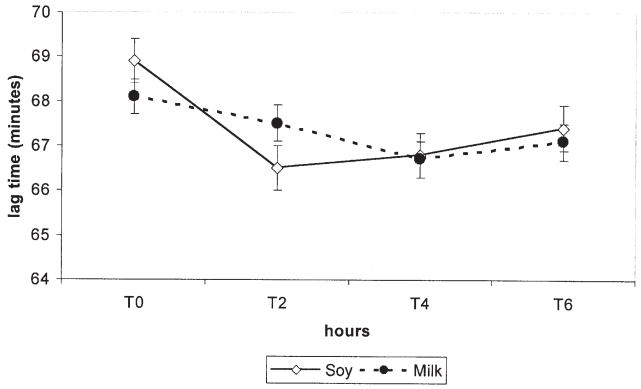

FIG. 1. Postprandial copper-induced LDL oxidation with a challenge meal containing soy and milk protein.

difference ($P > 0.05$) for LDL oxidation between the main effects (protein type, time points) or their interaction on LDL oxidation (lag time) (Fig. 1).

DISCUSSION

The results of our study suggest that biomarkers associated with the development of atherosclerosis (oxidative stress, inflammation, thrombosis, and hyperlipidemia) are minimally affected in healthy men consuming a controlled challenge meal high in total and saturated fat, with either 39 g soy or 40 g milk protein. The composition of the challenge meal was chosen to elicit a hyperlipidemic response to produce an atherogenic environment, while maintaining a constant polyunsaturated to saturated fat ratio between the two treatment days. As expected, we observed significant increases in plasma TG concentrations, which show that the high-fat meal did elicit a hyperlipidemic response. Previous research by Ceriello *et al*. (22) demonstrated that a high-fat meal can elicit meal-induced oxidative stress assessed *via ex vivo* copper-induced LDL oxidation in diabetic individuals. However, diabetes is often characterized by dyslipidemia with an elevated TG concentration and abnormally low HDL levels (70), and postprandial LDL in Type 2 diabetes are more easily oxidized than fasting LDL or from controls (59). The inclusion of healthy young men in our study may have precluded us from identifying a postprandial effect of soy consumption on oxidative stress, inflammation, coagulation, and hyperlipidemia.

In the current study we chose to use 39 g soy protein and 85 mg isoflavones based on previous studies that have evaluated the chronic intake of soy and oxidative stress (43,44,71). *In vitro* data have demonstrated that soy isoflavones act as antioxidants when incubated with plasma from fasted adult, human volunteers. For instance, LDL-oxidation resistance increases in the presence of at least 2.5 (72) to 50 μM (36) of genistein and daidzein. On the other hand, the antioxidant potential of soy *in vivo* remains unclear, as human intervention studies have produced mixed results (43,44,46,47,71). For instance, Jenkins *et al*. (44) investigated the effects of a soy-based breakfast cereal (36 g/d soy protein and 168 mg total isoflavones/100 g) consumed daily for 3 wk on blood lipids and oxidized LDL in 25 hyperlipidemic individuals. The treatment diet significantly reduced LDL oxidation (*ex vivo* assessment) compared with the control diet ($P < 0.05$) (44). In a separate study, Jenkins *et al*. (43) demonstrated with 31 hyperlipidemic men and postmenopausal women that a diet providing 33 g soy protein with 86 mg isoflavones for 1 mon along with a low-fat metabolic diet favorably reduced oxidative stress, as indicated by significantly decreased conjugated dienes ($P < 0.001$). These studies suggest that soy and isoflavone consumption can positively modify the *ex vivo* oxidative stress profile of hyperlipidemic individuals.

In a randomized cross-over study, normolipidemic men and women ($n = 24$) consumed a high isoflavone (15 g soy protein, 56.0 mg isoflavones) (HI) and a low isoflavone (15 g soy protein, 1.9 mg isoflavones) (LI) burger for 17 d each with a 25-d wash-out period. Plasma isoprostane concentrations [8-epi-prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)], an *in vivo* biomarker of whole-body lipid peroxidation, were significantly reduced following the HI diet as compared with the LI diet (326 ± 32 ng/mL vs. 405 ± 50 ng/mL, respectively; $P = 0.028$). Additionally, lag time for copper ion-induced LDL oxidation was significantly longer with the HI diet than with the LI diet (48 ± 2.4 and 44 ± 1.9 min, respectively; $P = 0.017$). Although the aforementioned studies provide support that moderate isoflavone intake (56–86 mg/d) is effective in reducing oxidative stress in healthy (71) and hyperlipidemic (43,44) adults, two recent, well-designed human feeding studies found soy consumption had no effect on *in vivo* and *ex vivo* biomarkers of oxidative stress (46,47).

In a double-blind, parallel arm study, Engelman *et al*. (46) provided 14 postmenopausal women with 40 g soy protein/d (86 mg isoflavone/d) and compared oxidative stress biomarkers with 14 women not consuming soy protein after 6 wk. No significant changes were found for any of the *in vivo* oxidative stress indexes (plasma protein carbonyls, oxidized LDL, and 8-epi-$PGF_{2\alpha}$). Similarly Vega-López and co-workers (47) reported no significant effect on oxidative stress biomarkers with the daily consumption of soy protein (25 g/1000 kcal) and soy isoflavone (50 mg/1000 kcal).

Although our study design differed from these investigations (46,47), in that it tested the effects of soy on postprandial (0–6 h post-meal), and not long-term (>1 wk), oxidative stress, our results were similar in that we found no effect of soy on oxidative stress measurements (e.g., LDL oxidation or lag time). On the other hand, investigators have suggested that soy protein may exert beneficial effects on oxidative systems and biomarkers not reported in our study, or others (46,47). Thus, investigations continue to explore the relationships between soy protein and oxidative stress.

Our study did not find a significant difference between treatment and control (soy vs. milk) in IL-6, CRP, or fibrinogen serum concentrations. Very few studies have examined the effect of soy and/or isoflavones on inflammatory (IL-6, CRP, TNF-α) or coagulation (fibrinogen) biomarkers (52–55), and none are postprandial (6-h post-meal) studies. For example, 41 hypercholesterolemic men and postmenopausal women consuming either a

low-fat control diet or a diet low (10 mg/d isoflavones) and high (70 mg/d isoflavones) in soy protein showed increased IL-6, but not CRP or TNF-α serum concentrations with the high-isoflavone diet (54). Dent *et al.* (53) reported no difference in fibrinogen, factor VII-c, or coagulant activity in perimenopausal women consuming a low (4 mg isoflavone), high (80 mg isoflavone), or control-whey diet isoflavones after 24 wk. These results agree with a shorter (17 d) feeding trial (55) and with a study of 45 healthy men consuming either lean meat or tofu (soy-containing product) for 1 mon (52). Tsai *et al*. (16) reported no significant changes in CRP at 2, 4, or 6 h after a standard high-fat meal. In contrast, Aljada and co-workers (24) showed that a mixed meal high in fat (50% total kcal) significantly ($P < 0.05$) increased plasma CRP at 3-h post-meal challenges. Although these studies suggest soy may have a minimal effect on inflammatory, fibrinolytic, or coagulation biomarkers, isolated soy protein and isoflavones have been shown to have favorable effects on endothelial function, as assessed with brachial artery reactivity measurements (56–58). Further, that IL-6 significantly decreased as a function of time during either meal challenge in our study indicates IL-6 is a sensitive biomarker in the postprandial period. Furthermore, it may be that significant IL-6 changes were not observed as a result of the timing of the blood draws. Future studies should include more frequent blood draws immediately after the meal consumption (e.g., every 30 min). The timing of blood draws for the current study was based on capturing the peak TG response to ensure an atherogenic environment in response to the high-fat meal (73). Additionally, CRP and fibrinogen may be more sensitive measures of systemic inflammation and hemostatic changes following a long-term dietary intervention and therefore would be minimally affected by a single meal challenge.

Our study had several limitations. Most importantly, the use of *ex vivo* LDL oxidation as our biomarker of oxidative stress limits our ability to evaluate the acute antioxidant effect of soy consumption. At the time this study was conducted, LDL oxidation was the primary biomarker used in soy and oxidative stress studies (43,44,71). To our knowledge, no studies have been published regarding the postprandial antioxidant effects of soy, but studies with other bioactive food components have found significant changes with the *ex vivo* LDL oxidation assessment (74–76). Thus, evidence exists to support the inclusion of LDL oxidation as an assessment parameter.

Although our meal challenges significantly increased TG concentrations, whether the hyperlipidemic state produced was enough to create an oxidative stress environment that could increase risk for atherosclerosis is not clear. Thus, additional biomarkers of antioxidant systems and responses such as F_2-isoprostane or glutathione peroxidase should be included in future studies. This would clarify whether the meal challenge has in fact induced prolonged oxidative stress *in vivo*, and if so, whether these are more sensitive biomarkers to use in postprandial oxidative stress studies.

An additional limitation is the significant difference in the saturated fat intake between the milk and soy treatment days for the 3 d prior to the meal challenges. That plasma TG concentrations significantly increased with both treatments suggests that the significant difference in saturated fat intake did not result in significantly different hyperlipidemic states between the two meal challenge days.

The final limitation of this study relates to the use of young, healthy men. An indication of their health status is the low serum CRP levels of the subjects. Future studies focused on evaluating the acute antioxidant effect of bioactive food components should consider the use of "non-healthy" or "at risk" participants. Engelman *et al*. (46) demonstrated a significant correlation between BMI and oxidative stress (serum $PGF_{2\alpha}$, $r = 0.33$; serum oxidized LDL, $r = 0.34$). Others have also shown that people with diabetes (77) or who smoke (78) have increased *in vivo* oxidative stress as reflected by elevated urinary isoprostane concentrations. Individuals with elevated oxidative stress and inflammatory profiles would be appropriate subjects to include in future studies.

Despite the controversy surrounding the antioxidant and anti-inflammatory effects of soy consumption, future studies continue to be warranted to elucidate whether long-term soy consumption can modify chronic disease risk by minimizing postprandial oxidative stress and inflammatory excursions. Future studies should include individuals with elevated oxidative stress and inflammatory profiles such as smokers and diabetic and/or obese individuals. This acute study did not elicit significant oxidative stress, inflammation, or hyperlipidemic changes between soy and milk protein.

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Oleate-Induced Formation of Fat Cells with Impaired Insulin Sensitivity

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ABSTRACT: Exogenous FA cause lipid accumulation in preadipocytes. We investigated whether the fat cells thus formed are metabolically distinct from adipocytes differentiated with standard methylisobutylxanthine, dexamethasone, and insulin (MDI) hormonal cocktail by comparing their expression of adipogenic genes, accumulation of TAG, lipogenesis, lipolysis, glucose uptake, and the effects of insulin on selected metabolic activities. Cells exposed to oleate began to accumulate TAG in parallel or prior to the induction of adipogenic genes, whereas cells treated with MDI expressed adipogenic genes before TAG accumulation. Oleate-treated fat cells also showed exaggerated basal lipolysis and weak response to insulin in both lipolysis regulation and glucose uptake. These findings were associated with increased basal phosphorylation of perilipin, increased Glut-1 but decreased Glut-4 expression, and reduced insulin-induced Akt phosphorylation. We suggest that this unique fat cell phenotype might be a mimetic of what can happen to fat cells formed *in vivo* under the influence of circulating FA and might be a useful model for *in vitro* studies of obesity-related insulin resistance in adipocytes.

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Exogenous FA have been shown to enhance preadipocyte differentiation, but the metabolic functions of fat cells thus formed have not been reported (1,2). We now show that fat cells formed by treating 3T3-L1 preadipocytes with oleic acid (oleate), while expressing adipocyte-specific genes to some extent, exhibit impaired insulin sensitivity in several important metabolic aspects as compared with MDI (methylisobutylxanthine, dexamethasone, and insulin)-induced adipocytes.

METHODS

Cells culture. 3T3-L1 Preadipocytes were treated with DMEM containing 10% FBS and 17 nM insulin +/– FA (oleate, palmitate, linoleate, 0.5 mM, all complexed to BSA at a 3:1 molar ratio). Control adipocytes were differentiated by treatment with an MDI hormone cocktail for 48 h and then switched to DMEM with 10% FBS and 17 nM insulin (3). All cells were incubated in low-glucose (5 mM) DMEM with 0.5% BSA for 24 h before metabolic experiments.

Cell cycle analysis. After treatments, cells were trypsinized and fixed with 70% ethanol. Samples were stained with propidium iodide (5 μg/mL in PBS with 20 μg/mL RNase) for 30 min at 37°C and then analyzed using FACScan flow cytometry (BD Biosciences, San Jose, CA).

Lipolysis, lipogenesis, glucose uptake, Western analysis, and statistical analysis. These steps were performed as previously described (4,5). RNA isolation and reverse transcription were done as described before (4). Real-time PCR was done by mixing 2 μL of first strand cDNA with an adequate amount of water and 10 μL TaqMan universal PCR master enzyme mix from Roche (Brunburg, NJ) using the Rotor-Gene 3000A instrument (Corbett Robotics, San Francisco, CA).

RESULTS AND DISCUSSION

Oleate-induced growth arrest and lipid accumulation. Figure 1A shows the changes in cell morphology before and after being treated with oleate, linoleate, and a mixture of palmitate, linoleate, and oleate (PLO, 1:1:1 molar ratio) for 4 d. All FA were added at 0.5 mM with 0.14 mM BSA. Palmitate alone added at the same concentration was found to induce apoptosis (data not shown). For comparison, a microphotograph of cells differentiated with standard MDI protocol is also shown (7 d post-differentiation). These results indicated that all the exogenous FA caused intracellular lipid accumulation, with oleate being the most efficient in forming large, coalescent lipid droplets. Since oleate is mitogenic in selected cell types (6–8), we performed fluorescence-activated cell sorting (FACS) analysis to assess whether this might occur in our cell systems. As shown in Figure 1B, a similar cell population distribution pattern was found for postconfluent preadipoctyes before and after being treated with oleate or MDI: ~75–80% at G1/G0, ~15% at G2/M and ~5% at S phase. Such a distribution pattern is typical for growth-arrested fibroblasts (9). To further test whether the cells maintained at a growth-arrested state after being treated with oleate, we performed real-time PCR analysis for the expression of G0S2 gene, a novel marker for growth arrest and differentiation in 3T3-L1 cells (10). As shown in Figure 1C, treatment with FA or MDI increased G0S2 mRNA substantially as compared with growing preadipocytes. Together, these data suggest that treating 3T3-L1 preadipocytes with oleate alone or in combination with other FA resulted in the formation of fat cells that mimic

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Abbreviations: Akt, protein kinase B; aP2, FA binding protein; C/EBPα, CCAAT/enhancer-binding protein-α; DGAT-2, DAG acyltransferase-2; FACS, fluorescence-activated cell sorting; GPDH, glycerol-3-phosphate dehydrogenase; MDI, methylisobutylxanthine, dexamethasone, and insulin; PCR, polymerase chain reaction; PLO, palmitate, linoleate, and oleate; PPARγ, peroxisome proliferator-activated receptor-γ.

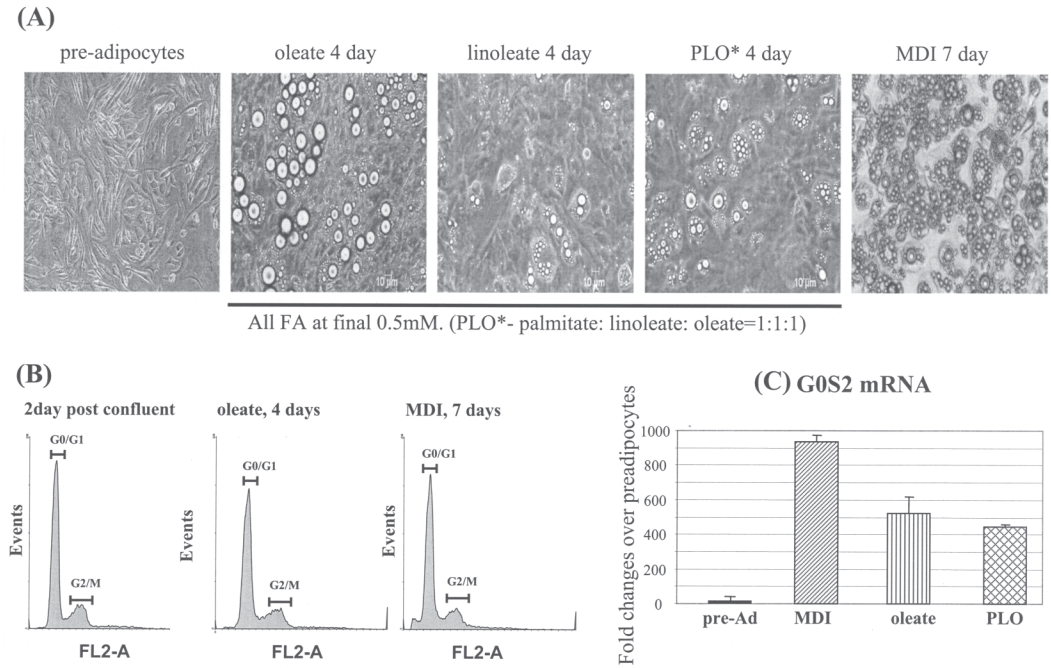

FIG. 1. Effects of FA on cell morphology and growth arrest. (A) Morphological lipid accumulation; (B) fluorescence-activated cell sorting (FACS) analysis of cell cycle population distribution; (C) steady-state expression of G0S2 mRNA in 3T3-L1 preadipocytes (pre-Ad) before and after being treated with FA [oleate or palmitate-linoleate-oleate (PLO)] or the methylisobutylxanthine, dexamethasone, and insulin (MDI) cocktail. FL2-A, propidium iodide.

the adipocytes differentiated with the standard MDI protocol. We then further characterized this new type of fat cells at the molecular and metabolic levels.

Expression of adipogenic genes. As shown in Figure 2A, oleate induced a gradual increase in expression of the key adipogenic transcription factors, peroxisome proliferator-activated receptor-γ (PPARγ) and C/EBPα (CCAAT/enhancer-binding protein-α), in preadipocytes. After being normalized to the maximal expression level at maturation (day 7), the time courses of PPARγ and C/EBPα induction in cells treated with oleate were similar to that in cells treated with MDI, except that PPARγ expression was induced to a greater extent in the former during the early phase (day 0–2, Fig. 2A). Figure 2B shows that oleate also induced expression of selected differentiation marker genes, including FA binding protein (aP2), CD36, DAG acyltransferase-2 (DGAT-2), and glycerol-3-phosphate dehydrogenase (GPDH). Compared with MDI, oleate had a similar induction of GPDH, two- to threefold less CD36 and DGAT2 but two- to threefold more *aP2*. This suggests that fat cells formed after the two different treatments were similar but not identical and that MDI might be a more potent inducer for adipogenesis. Oleate, on the other hand, might specifically increase steady-state aP2 mRNA *via* alternative mechanisms as previously reported (11). Treatment with a combination of PLO gave a similar induction as that induced by oleate alone.

TAG accumulation. In the absence of oleate or MDI, little TAG was found in preadipocytes (Fig. 2C), consistent with a previous report that insulin alone does not induce adipogenesis (12). Cells treated with oleate began to accumulate TAG within 24 h, which reached a plateau at day 3–4 (Fig. 2C). On the other hand, TAG was undetectable in MDI-treated cells in the first 3 d but began to accumulate rapidly thereafter. These results indicate that TAG accumulation was tightly associated with adipogenesis in MDI-treated cells but not in oleate-treated cells.

Metabolic characterization. As shown in Figure 3A, cells treated with oleate or MDI both showed a higher basal lipogenesis rate as compared with the untreated preadipocytes. Insulin further stimulated lipogenesis two- to threefold (Fig. 3A), indicating that the insulin-sensitive lipogenic pathway was developed to a similar extent in both cell types. On the other hand, Figure 3B shows that oleate-treated cells had a greater basal glucose uptake than the MDI-treated cells. However, glucose uptake in the oleate-treated cells was not responsive to insulin stimulation at or near physiological concentration (170 nM) and only moderately responsive at supraphysiological concentration (1.7 μM), as compared with cells treated with MDI that showed a 3.5-fold increase in response to 170 nM insulin but with no further increase when insulin was increased to 1.7 μM. This difference suggests that the insulin signaling pathways were not activated properly in oleate-treated cells. Figure 3C shows that oleate-treated cells also had a greater basal lipolysis as compared with cells treated with MDI. However, isoproterenol-stimulated lipolysis was blunted, indicating dampened sensitivity to β-agonist as compared with that in the MDI-treated cells. Insulin also had no effect on isoproterenol-stimulated lipolysis in oleate-treated cells although it greatly suppressed that in the MDI-treated cells (Fig. 3C).

Altered metabolic characteristics are associated with

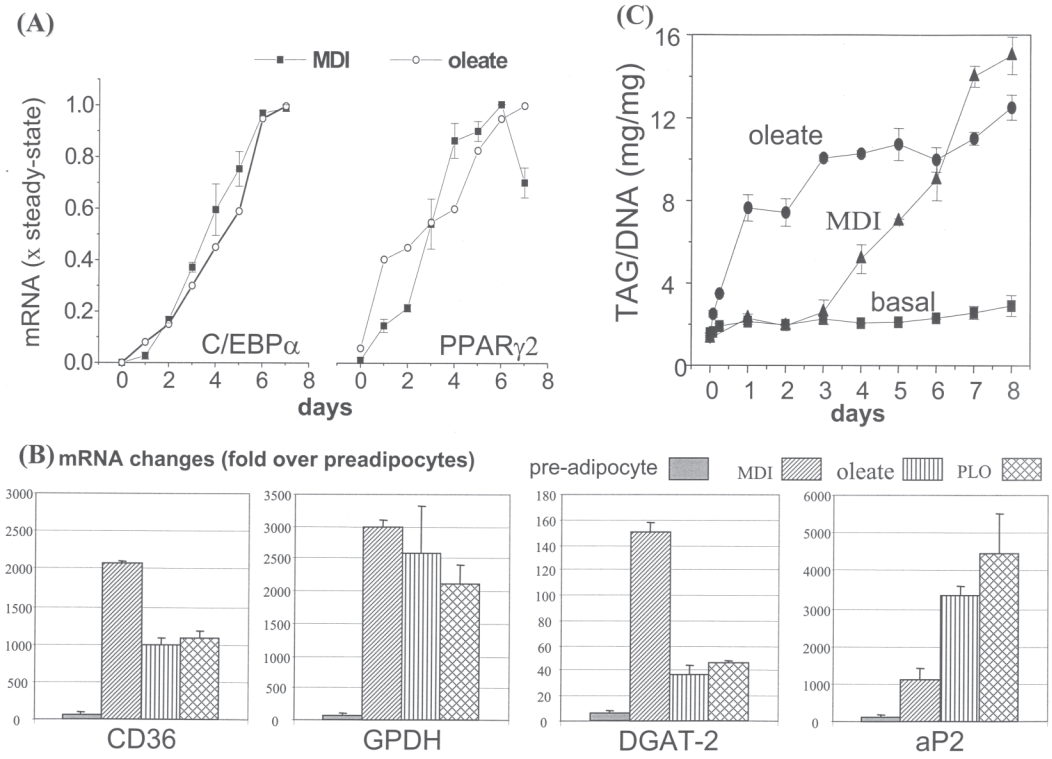

FIG. 2. Effects of FA on adipogenic gene expression and TG storage. (A) Time-dependent expression of CCAAT/enhancer-binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ) in preadipocytes treated with oleate or MDI, normalized to the steady-state expression levels on day 7; (B) time-dependent TAG accumulation (mean ± SE, $n = 3$); (C) mRNA expression of selected adipogenesis-related genes on day 7. GPDH, glycerol-3-phosphate dehydrogenase; DGAT-2, DAG acyltransferase-2; aP2, FA binding protein; for other abbreviations see Figure 1.

changes in the expression or phosphorylation of key functional proteins. First, we measured the expression/phosphorylation of perilipin, a protein that coats the lipid droplets in fat cells and facilitates lipolysis when phosphorylated (13). Consistent with the exaggerated basal lipolysis (Fig. 3C), we found that oleate-treated cells had a higher level of basal perilipin phosphorylation than MDI-treated cells (Fig. 4A). Second, we measured the expression of Glut-1 and Glut-4, the transporter proteins

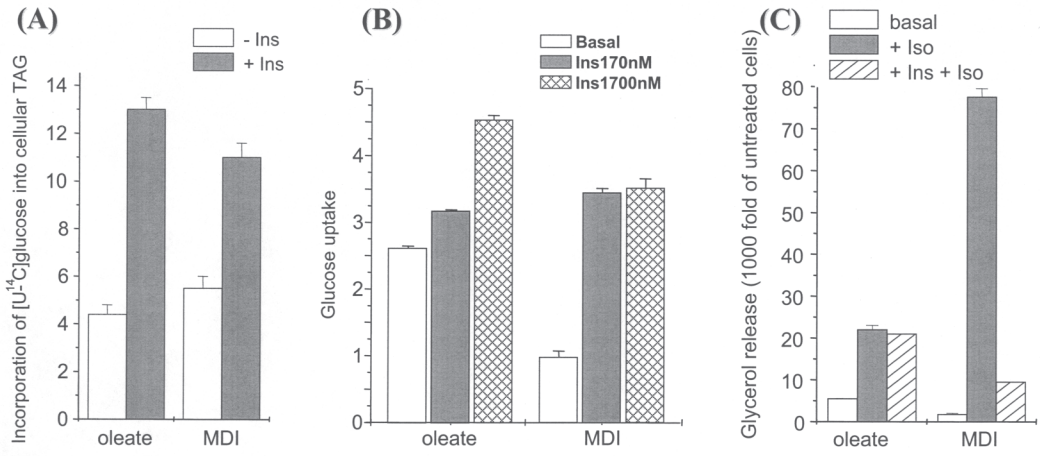

FIG. 3. Effects of oleate on metabolic functions and cellular response to insulin. (A) Incorporation of [U-^{14}C] glucose into cellular lipids, (B) uptake of 2-deoxy [2,6-^{3}H] glucose, (C) glycerol release, with or without insulin (170 nM, Ins) or isoproterenol (0.5 μM, Iso). Results are presented as fold changes over the untreated cells, for which the basal rates of lipogenesis, glucose uptake, and glycerol release were 0.82 pmol/μg DNA/min, 0.06 nmol/μg DNA/min, and 0.8 pmol/μg TAG/min, respectively (mean ± SE, $n = 3$, Duncan's test). For abbreviation see Figure 1.

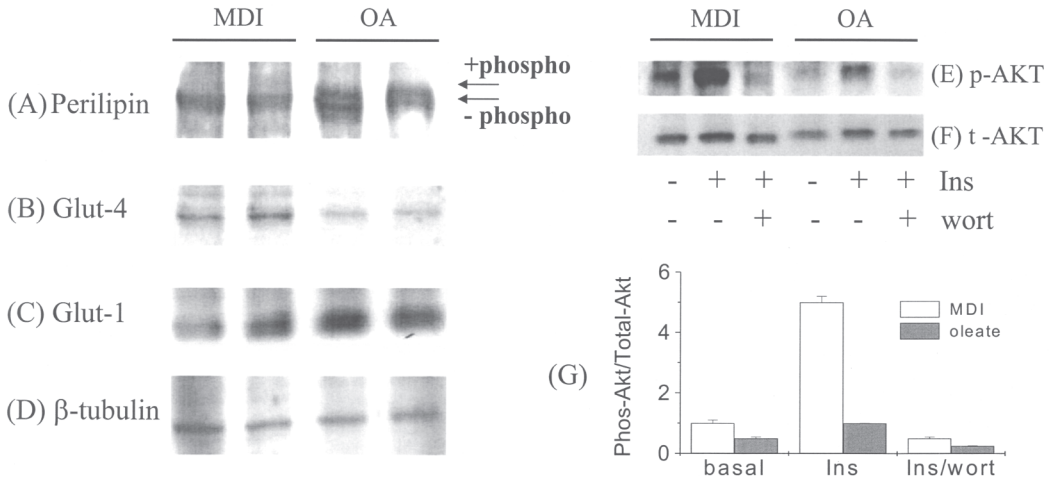

FIG. 4. Western analysis of selected proteins as labeled. Cells were prepared as in Figure 2 and used directly (A–D) or after incubation with insulin (170 nM, 15 min) with or without wortmanin (500 nM, wort) (E,F). Cells were lysed using RIPA buffer containing inhibitors for proteases and phosphatases, and 20 μg crude protein lysate of each sample was loaded for electrophoresis. Results are representative of three independent experiments. OA, oleate; p-AKT, phosphorylated protein kinase B; t-AKT, total protein kinase B protein; for other abbreviations see Figures 1 and 3.

that control the basal and insulin-stimulated glucose uptake, respectively (12). As shown in Figures 4B and 4C, oleate-treated cells had a greater expression of Glut-1 but less of Glut-4 compared with MDI-treated cells. This is consistent with the elevated basal, but dampened insulin-stimulated, glucose uptake in the former (Fig. 3B). Finally, we showed that insulin-stimulated protein kinase B (Akt) phosphorylation was also dampened in oleate-treated cells (~twofold), compared with a more sensitive response in MDI-treated cells (~fivefold) (Fig. 4E–G). This might also contribute to the poor insulin sensitivity with regard to lipolysis and glucose uptake in oleate-treated cells, based on the pivotal role of Akt in control of insulin signaling (14). The insulin-stimulated lipogenesis, on the other hand, appeared to be normally developed in these cells (Fig. 3A).

In summary, we showed that oleate induced the formation of a unique fat cell phenotype that exhibited some of the features of adipocytes, as shown by the expression of adipogenic genes, active lipogenesis, and lipolysis. However, these cells were also relatively insensitive to insulin and β-agonist. It is almost certain that they also differ from normal adipocytes in other functional aspects that still remain to be determined. Because elevation of blood FA concentration is common after a high-fat diet, and high-fat diets are known to be associated with increased fat cell formation *in vivo* (15–18), it is possible that fat cells thus formed may bear similar features to those of oleate-treated preadipocytes *in vitro*, with augmented fat storage but impaired hormonal sensitivity. In this regard, this work might provide a useful *in vitro* model for studying the effects of FA on adipose tissue development.

ACKNOWLEDGMENT

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Coexistence of Phosphatidylcholine-Specific Phospholipase C and Phospholipase D Activities in Rat Cerebral Cortex Synaptosomes

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ABSTRACT: DAG derived from phosphatidylcholine (PtdCho) acts as a lipid second messenger. It can be generated by the activation of phospholipase D (PLD) and the phosphatidic acid phosphohydrolase type 2 (PAP2) pathway or by a PtdCho-specific phospholipase C (PtdCho-PLC). Our purpose was to study PtdCho-PLC activity in rat cerebral cortex synaptosomes (CC Syn). DAG production was highly stimulated by detergents such as Triton X-100 and sodium deoxycholate. Ethanol and tricyclodecan-9-yl-xanthate potassium salt decreased DAG generation by 42 and 61%, respectively, at 20 min of incubation. These data demonstrate that both the PLD/PAP2 pathway and PtdCho-PLC contribute to DAG generation in CC Syn. PtdCho-PLC activity remained located mainly in the synaptosomal plasma membrane fraction. Kinetic studies showed K_m and V_{max} values of 350 μM and 3.7 nmol DAG × (mg protein × h)$^{-1}$, respectively. Western blot analysis with anti-PtdCho-PLC antibody showed a band of 66 KDa in CC Syn. Our results indicate the presence of a novel DAG-generating pathway in CC Syn in addition to the known PLD/PAP2 pathway.

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Phosphatidylcholine (PtdCho) is the most abundant class of glycerophospholipids in mammalian cell membranes, where it plays key roles in membrane structure, cell death, and cellular signaling (1). This phospholipid is also an important source of second lipid messengers such as lysophosphatidylcholine, phosphatidic acid (PtdOH), arachidonic acid, and DAG.

PtdCho hydrolysis can be elicited by growth factors, cytokines, neurotransmitters, hormones, and other extracellular signals (2–5). In response to several stimuli, DAG can be generated from PtdCho by subsequent activation of phospholipase D (PLD) and PtdOH phosphohydrolase type 2 (PAP2) or by a PtdCho-specific phospholipase C (PtdCho-PLC) (2,6). In contrast to DAG generated from phosphatidylinositol (4,5) bisphosphate ($PtdInsP_2$) by PtdIns-PLC activity, the wave of DAG elicited from PtdCho hydrolysis is generated more slowly and proceeds without elevation of intracellular Ca^{2+}. PtdCho-PLC activation might be related to a sustained activation of Ca^{2+}-independent protein kinase C (PKC) isoforms suggesting its involvement in slow and extended cell responses such as proliferation and differentiation (6,7).

PtdCho-PLC activity has been previously described in several rat tissues (8) as well as in rat platelets (9), and it has been isolated and characterized in bull seminal plasma (10). PtdCho-PLC activity is involved in many cellular events such as glutamate-induced nerve cell death (11) and in Fas-induced apoptosis (12,13). PtdCho-PLC also can be activated during cell mitogenic responses that are triggered by platelet-derived growth factor (14,15), thromboxane A_2 (16), and lipopolysaccharide (LPS) (17), leading to the activation of the mitogen-activated protein kinase pathway. Furthermore, PtdCho-PLC seems to be acting downstream Ras but upstream Raf-1 during mitogenic signal transduction (18,19).

Studies from our laboratory demonstrated the presence of the PLD/PAP2 pathway and its regulation by insulin in rat cerebral cortex (CC) synaptosomes (Syn) (20,21). Mammalian PLD isoforms (PLD1, PLD2) are present in neurons and in glial cells, and these enzymes are implicated in basic cellular functions such as vesicular trafficking as well as in brain development (22). However, the PtdCho-PLC pathway has not been thoroughly studied in the central nervous system (CNS), and no evidence has been reported to date about its presence in synaptic endings and its involvement in signal transduction events. In view of its wide distribution and its implication in such diverse cell responses, the aim of this work was to analyze PtdCho-PLC activity in rat CC Syn.

MATERIALS AND METHODS

Wistar-strain adult rats (4 mon old) were kept under constant environmental conditions and fed on a standard pellet diet *ad libitum* until decapitation.

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Abbreviations: Ab, antibody; CC, cerebral cortex; CM, crude mitochondrial pellet; CNS, central nervous system; D609, tricyclodecan-9-yl-xanthate potassium salt; DOC, sodium deoxycholate; DPPtdCho, dipalmitoylphosphatidylcholine; LPS, lipopolysaccharide; NK, natural killer; PAP2, phosphatidic acid phosphohydrolase type 2; PKC, protein kinase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdCho-PLC, phosphatidylcholine-specific phospholipase C; PtdEth, phosphatidylethanol; $PtdInsP_2$, phosphatidylinositol (4,5) bisphosphate; $PtdInsP_2$-PLC, phosphatidylinositol (4,5) bisphosphate phospholipase C; PtdOH, phosphatidic acid; PVDF, polyvinylidene difluoride; RHC80267, 1,6-bis(cyclohexyloximinocarbonylamino)hexane; SM, sphingomyelin; SMS, sphingomyelin synthase; Sol, soluble fraction; SPM, synaptosomal plasma membrane; Syn, synaptosomes, synaptosomal fraction; TH, total homogenate; TNF, tumor necrosis factor; T X-100, Triton X-100.

1-[^{14}C]Palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]DPPtdCho) (111 mCi/mmol), 1,2-dipalmitoyl-*sn*-glycero-3-phospho[methyl-^{3}H]choline ([^{3}H-choline] DPPtdCho) (43 Ci/mmol), and Omnifluor were obtained from New England Nuclear-DuPont (Boston, MA). Triton X-100 (T X-100), sodium deoxycholate (DOC), tricyclodecan-9-yl-xanthate potassium salt (D609), and 1,6-bis(cyclohexyloximinocarbonylamino)hexane (RHC80267) were obtained from Sigma-Aldrich (St. Louis, MO). Polyclonal anti-PtdCho-PLC antibody (Ab) raised against *Bacillus cereus* PtdCho-PLC was generously provided by Dr. Howard Goldfine, University of Pennsylvania (Philadelphia, PA). All other chemicals were of the highest purity available.

Preparation of synaptosomal fraction. Total homogenates were prepared from the CC of 4-mon-old rats. Rats were killed by decapitation and CC was immediately dissected (2–4 min after decapitation). All proceedings were in accordance with *Principles of Use of Animals and Guide for the Care and Use of Laboratory Animals* (National Institutes of Health regulation).

The synaptosomal fraction (Syn) was obtained as previously described by Cotman (23) with slight modifications (20). Briefly, CC homogenate was prepared in the following way: 20% (wt/vol) in a buffer containing 1 mM EDTA, 10 mM HEPES buffer (pH 7.4) in the presence of 1 mM DTT, 2 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin, and 0.1 mM PMSF.

The CC homogenate was centrifuged at 1,800 × *g* for 7.5 min using a JA-21 rotor in a Beckman J2-21 centrifuge, and the supernatant was carefully poured into another tube. The supernatant was subsequently centrifuged at 15,000 × *g* for 20 min to obtain the crude mitochondrial pellet (CM). The CM was washed and resuspended with 3 mL of the isolation medium and loaded onto a 8.5% Ficoll medium (6 mL) and 13% Ficoll medium (6 mL) discontinuous gradient. The Ficoll solutions were prepared in the isolation medium. The sample loaded onto the discontinuous gradient was centrifuged at 120,000 × *g* for 30 min using a SW 28.1 rotor in a Beckman L5-50 ultracentrifuge. The myelin fraction band was at the interface between the isolation medium and the 8.5% Ficoll medium, the Syn band at the interface between the 8.5% and the 13% Ficoll medium, and the free mitochondrial fraction was the pellet below the 13% Ficoll medium. Syn was centrifuged at 33,000 × *g* for 20 min using a JA-21 rotor in a Beckman J2-21 centrifuge and resuspended in the assay buffer.

Preparation of Syn plasma membrane (SPM). SPM isolation was performed according to a protocol previously described by Igbavboa *et al.* (24) with slight modifications. Briefly, the Syn fraction from one CC was resuspended in 4 mL of cold lysis buffer (5 mM Tris buffer, pH 8.5). The lysed suspension was vortex-mixed vigorously and incubated for 1 h at 4°C with repeated vortex mixing every 15–20 min. After 1 h the suspension was centrifuged at 33,000 × *g* for 30 min using a JA-21 rotor in a Beckman J2-21 centrifuge. The soluble fraction (Sol) from lysed Syn was collected; the pellet was resuspended in 4 mL of cold distilled water, layered onto 12 mL of 0.75 M sucrose containing 10 mM HEPES and 0.25 mM EDTA (pH 7.4), and centrifuged at 73,000 × *g* for 30 min using an SW 28.1 rotor in a Beckman L5-50 ultracentrifuge. The SPM suspension at the interface was removed and centrifuged at 33,000 × *g* for 30 min in a JA-21 rotor in a Beckman J2-21 centrifuge. The SPM pellet was resuspended in the assay buffer. To establish the purity of Syn subfractions, a 5′ nucleotidase assay was performed according to Widnell and Unkeless (25).

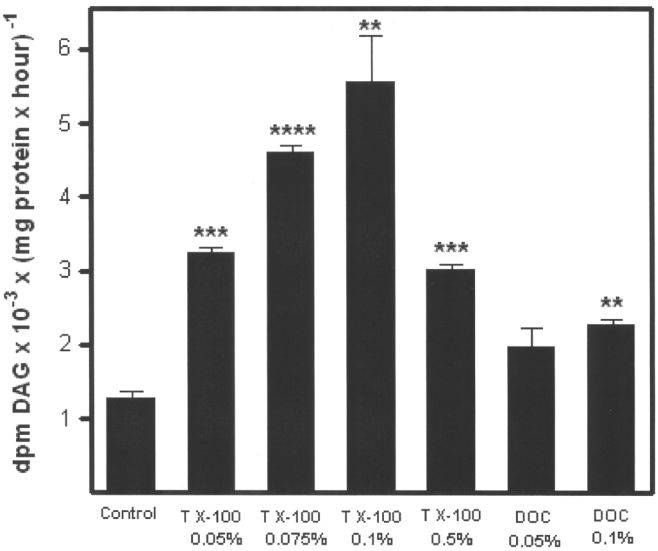

FIG. 1. Effect of detergents on synaptosomal DAG generation from phosphatidylcholine (PtdCho). Rat cerebral cortex (CC) synaptosomes (Syn) were incubated in a buffer containing 0.1 M Tris (pH 7.2) in the presence of 0.125 mM dipalmitoylphosphatidylcholine (DPPtdCho) (40,000 dpm of [^{14}C]DPPtdCho per assay) and different concentrations of Triton X-100 (T X-100; 0.05, 0.075, 0.1, and 0.5%) or sodium deoxycholate (DOC; 0.05 and 0.1%) as described in the Materials and Methods section. After 20 min, the enzyme reaction was stopped and lipids were extracted and isolated as described in the Materials and Methods section. Results are compared with the control condition without detergent, and the enzyme activity is expressed as dpm DAG × 10^{-3} × (mg protein × h)$^{-1}$. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples (**$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$).

Determination of PtdCho-PLC activity. PtdCho hydrolysis by PtdCho-PLC was determined using lipid vesicles containing [^{14}C]DPPtdCho and cold DPPtdCho to yield 40,000 dpm and 0.125 mM per assay. These lipid vesicles (100 μL) were added to 100 μL of Syn, SPM, or Sol (150 μg of protein) in a final volume of 200 μL in a buffer containing 0.1M Tris (pH 7.2). The reaction was incubated at 37°C for 5 or 20 min and stopped by the addition of 5 mL of chloroform/methanol (2:1, vol/vol). Blanks were prepared identically, except that membranes were boiled for 5 min before being used. Lipids were extracted and separated as described below.

Determination of PLD activity. To assay PLD activity, transphosphatidylation reaction was measured in Syn and SPM. Lipid vesicles were prepared as described above and the assay was conducted in the presence of 2% ethanol. The reaction was incubated at 37°C for 5 or 20 min and stopped by the

addition of 5 mL of chloroform/methanol (2:1, vol/vol). Lipids were extracted and separated as described below.

Determination of sphingomyelin synthase (SMS) activity. SMS activity was assayed as described for PtdCho-PLC, but lipid vesicles were prepared with [^{3}H-choline]-DPPtdCho as labeled substrate. The reaction was incubated at 37°C for 5 or 20 min and stopped by the addition of 5 mL of chloroform/methanol (2:1, vol/vol). Lipids were extracted and separated as described below.

Extraction and isolation of lipids. Lipids were extracted according to Folch *et al.* (26). Briefly, the lipid extract was washed with 0.2 vol of 0.05% $CaCl_2$ and the lower phase was obtained after centrifugation at 900 × *g* for 5 min. Neutral lipids (MAG, DAG, and FFA) were then separated by 1-D TLC using silica gel G plates (Merck, Darmstadt, Germany) in a mobile phase consisting of hexane/diethyl ether/acetic acid (50:50:2.6, by vol). PtdCho was retained at the spotting site. Lipids were visualized by exposure of the plate to iodine vapors. DAG spots were scraped off for counting by liquid scintillation.

Phosphatidylethanol (PtdEth) was separated by 1-D TLC on silica gel H (Merck) and developed with chloroform/methanol/acetone/acetic acid/water (50:15:15:10:5, by vol) up to 70% of the plate. Then the plate was rechromatographed up to the top using hexane/diethyl ether/acetic acid (70:30:2.6, by vol) as described by Salvador and Giusto (27). Radioactivity of lipid spots was determined as previously described.

Sphingomyelin (SM) was separated by 1-D TLC on silica gel H (Merck) and developed with chloroform/methanol/27% ammonia (65:25:5, by vol). Radioactivity of lipid spots was determined as previously described.

Western blot assay of PtdCho-PLC. Syn samples were resuspended in Laemmli buffer and boiled for 3 min. Protein (60 µg) was loaded and resolved in a 10% SDS-PAGE following Laemmli (28) and transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot cell electroblotter (Bio-Rad Life Science Group, Hercules, CA) for 1 h. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl) containing 0.1% Tween-20 (2 h at room temperature) and then incubated with rabbit polyclonal anti-PtdCho-PLC Ab overnight at 4°C. Immunoreactions were detected with polyclonal horseradish peroxidase conjugated to total antirabbit Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with enhanced chemiluminescence substrates (New England Nuclear-Dupont).

Other methods. Protein concentration was determined according to Bradford (29).

Statistical analysis. Statistical analysis was performed using Student's *t*-test.

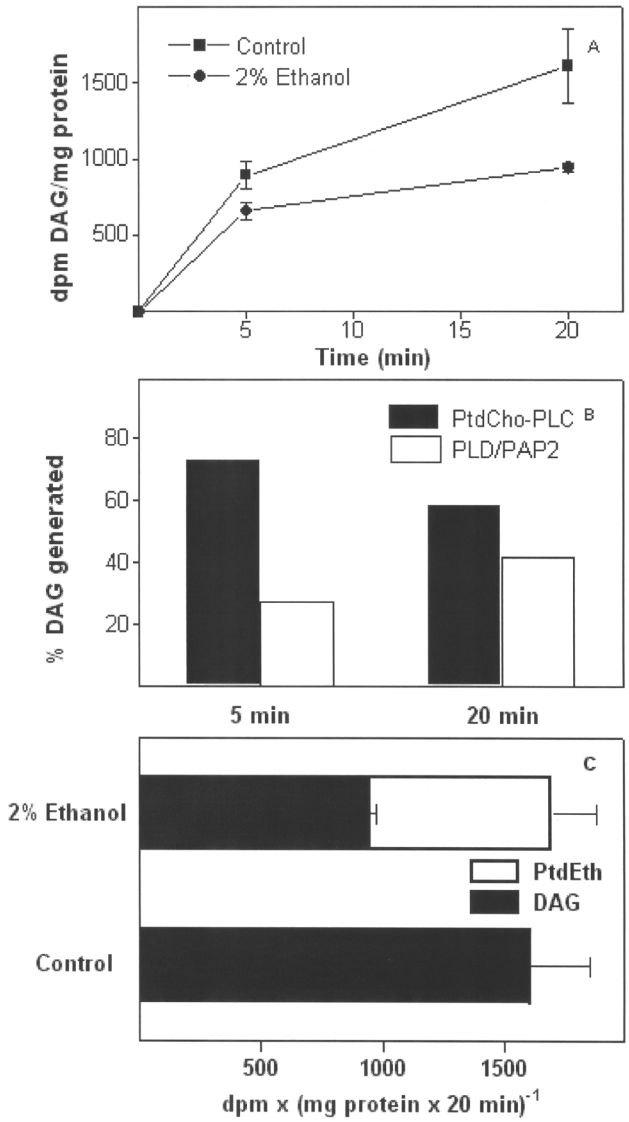

FIG. 2. (A) Contribution of PtdCho-PLC (PtdCho-specific phospholipase C) and phospholipase D/phosphatidic acid phosphohydrolase type 2 (PLD/PAP2) pathways to synaptosomal DAG generation. The enzyme assay was carried out as described in Figure 1 in a buffer containing 0.1% T X-100 with 2% ethanol and without ethanol (control condition). DAG generation was evaluated as a time function (5 and 20 min), and results are expressed as dpm DAG/mg protein. (B) Contribution of PtdCho-PLC and PLD/PAP2 pathways to synaptosomal DAG formation as a time function. Results are expressed as the percentage of the total DAG generated. (C) Synaptosomal phosphatidylethanol (PtdEth) formation. PtdEth production was measured as described in the Materials and Methods section. DAG and PtdEth levels obtained in Syn incubated with 2% ethanol are compared with DAG levels obtained under control condition at 20 min incubation. Results are expressed as dpm × (mg protein × 20 min)$^{-1}$. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples. For other abbreviations see Figure 1.

RESULTS

Effect of detergents on DAG generation in CC Syn. The effect of detergents such as DOC and T X-100 on synaptosomal DAG generation was studied. Syn were incubated in the presence of 0.125 mM DPPtdCho (40,000 dpm of [^{14}C]DPPtdCho per assay) and in different concentrations of T X-100 (0.05, 0.075, 0.1, and 0.5%) or DOC (0.05 and 0.1%) as described in the Materials and Methods section. The effect of these tensioactive

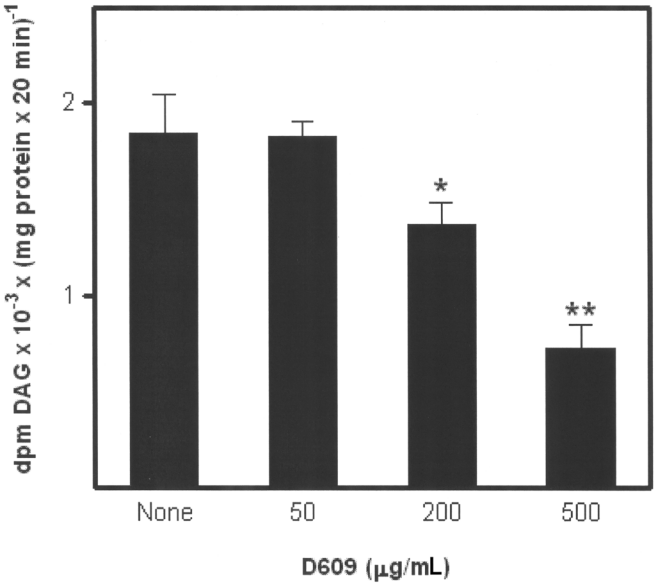

FIG. 3. Effect of tricyclodecan-9-yl-xanthate potassium salt (D609) on synaptosomal DAG formation. Syn were preincubated at 4°C for 20 min in the presence of different concentrations of D609 (50, 200, and 500 μg/mL). The enzyme reaction was carried out as described in Figure 2. Results are compared with the control condition, in which samples were preincubated without the inhibitor, and are expressed as dpm DAG × (mg protein × 20 min)$^{-1}$. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples (*$P < 0.025$; **$P < 0.01$). For abbreviation see Figure 1.

agents on synaptosomal DAG generation is shown in Figure 1. Both detergents stimulated DAG generation at all concentrations assayed when the activity was compared with the control condition (without any detergent). The major DAG generation was observed with 0.1% T X-100: DAG formation increased by 330% with respect to the control condition. At the same concentration, DOC increased DAG generation by only 77% with respect to the control condition. Based on these results, all subsequent experiments were conducted in the presence of 0.1% T X-100.

Contribution of PtdCho-PLC and PLD/PAP2 pathways to synaptosomal DAG generation. To study the contribution of PtdCho-PLC and PLD/PAP2 pathways to synaptosomal DAG generation, ethanol was used as a marker of PLD activity. The enzyme assay was conducted in the presence of 2% ethanol, and DAG generation was evaluated as a time function (5 and 20 min) and compared with the control condition (without ethanol) (Fig. 2A).

Figure 2B shows the contribution of PtdCho-PLC and PLD/PAP2 pathways to DAG formation in CC Syn. At 5 min incubation, 73% of the total DAG generated is originated by PtdCho-PLC activity while the remaining 27% is generated by the PLD/PAP2 pathway. At 20 min incubation, 58% of DAG is generated by PtdCho-PLC activity and 42% is from the PLD/PAP2 pathway, respectively. In the presence of ethanol, DAG and PtdEth levels correlated to total DAG levels generated under control condition (Fig. 2C), indicating that PtdOH generated by PLD was converted to PtdEth.

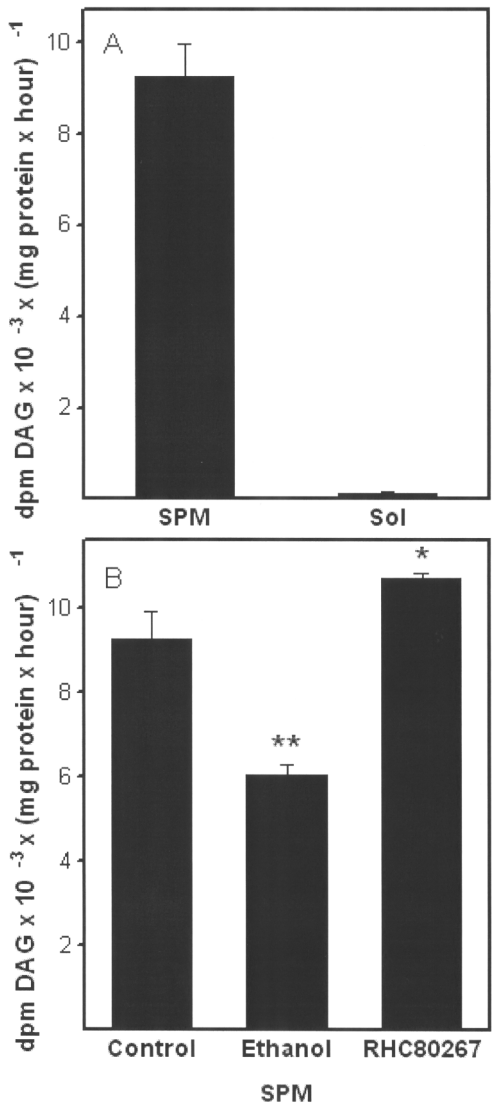

FIG. 4. (A) Localization of PtdCho-PLC activity. DAG formation was measured in the synaptosomal plasma membrane (SPM) and soluble fractions (Sol) obtained from Syn. SPM and Sol were isolated as described in the Materials and Methods section, and the enzyme reaction was carried out as described in Figure 2. (B) Effect of ethanol and 1,6-bis(cyclohexyloximinocarbonylamino)hexane (RHC80267) on DAG generation in SPM. DAG formation was measured under control conditions and in the presence of either 2% ethanol or 30 μM RHC80267 using SPM as the enzyme source. Results are expressed as dpm DAG × 10^{-3} × (mg protein x h)$^{-1}$. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples (*$P < 0.025$; **$P < 0.01$). For other abbreviations see Figure 1.

Effect of D609 on DAG formation. To evaluate the effect of the PtdCho-PLC inhibitor D609, Syn were preincubated at 4°C for 20 min in the presence of increasing concentrations of D609 (50, 200, and 500 μg/mL) before starting the enzyme reaction. Results in Figure 3 show that D609 inhibited DAG formation in a dose-dependent manner: 50 μg/mL had no effect on DAG generation whereas 200 μg/mL decreased DAG formation by

TABLE 1
Protein and 5′ Nucleotidase Distribution in Total Homogenate, Synaptosomes, Synaptosomal Plasma Membrane, and Soluble Fractions Prepared from Rat Cerebral Cortex[a]

Subcellular fraction	mg protein/g CC	5´ Nucleotidase activity	Nucleotidase activity/ TH nucleotidase activity
TH	145.8	352 ± 56	1
Syn	12.02	684 ± 96	1.94
SPM	2.56	1500 ± 2.4	4.26
Sol	8.7	40 ± 16	0.11

[a]Subcellular fractions were prepared as described in the Materials and Methods section. The 5′ nucleotidase activity is expressed as nmol Pi × (mg protein × h)$^{-1}$. Data are the mean ± SD of four samples. TH, total homogenate; Syn, synaptosomes; SPM, synaptosomal plasma membrane; Sol, soluble fraction; Pi, inorganic phosphorus.

26% and 500 μg/mL decreased DAG formation by 61% with respect to the control condition (without the inhibitor).

Localization of PtdCho-PLC activity. For determining the localization of PtdCho-PLC activity, PtdCho hydrolysis was measured in the plasma membrane fraction and in the Sol, obtained after the lysis of Syn. SPM and Sol were isolated as described in the Materials and Methods section, and the enzyme reaction was carried out as previously described.

Figure 4A shows that PtdCho-PLC activity was present in the SPM fraction whereas enzyme activity was negligible in Sol. These results are evidence that PtdCho-PLC is located in the SPM fraction. To evaluate the contribution of the PLD/PAP2 pathway to DAG formation in SPM, enzyme assays were carried out in the presence of 2% ethanol (Fig. 4B). At 20 min incubation, ethanol inhibited DAG generation by 35%. These results demonstrated that 65% of DAG was generated by PtdCho-PLC activity and 35% was from PLD/PAP2 pathway in the plasma membrane fraction.

As DAG lipase, which is a very active enzyme, is also located in the plasma membrane, DAG formation was evaluated in the presence of the DAG lipase inhibitor, RHC80267. DAG formation in the presence of 30 μM RHC80267 was increased by 16% in SPM with respect to the control condition (Fig. 4B). The activity of 5′ nucleotidase was measured in Syn, SPM, Sol, and total homogenate (TH), for evaluating purity of fractions (Table 1). The specific activity of 5′ nucleotidase in SPM was 4.26-fold higher than the specific activity present in TH.

Determination of PtdCho-PLC K_m and V_{max}. To determine PtdCho-PLC K_m and V_{max} values, enzyme reactions were carried out in the presence of increasing concentrations of exogenous DPPtdCho. DAG generation was measured using 50, 75, 125, 200, 300, 500, 700, and 900 μM of DPPtdCho (150,000 dpm per assay) in the presence of 2% ethanol. DAG generation increased linearly up to 200 μM DPPtdCho; at higher concentrations PtdCho-PLC showed a saturation behavior (Fig. 5A). K_m and V_{max} values calculated from a Lineweaver-Burk plot were 350 μM and 3.7 nmol DAG × (mg protein × h)$^{-1}$, respectively (Fig. 5B).

Detection of synaptosomal PtdCho-PLC by Western blot assay. Synaptosomal proteins were resolved in a 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was then incubated with a polyclonal Ab raised against *B. cereus* PtdCho-PLC (Fig. 6). *Clostridium perfringens* PtdCho-PLC (line A, Fig. 6) was used as a positive control for the Ab since the N-terminal domain of this protein shows structural similarity to *B. cereus* PtdCho-PLC (30). CC Syn from adult rats (line B) showed a band corresponding to 66 kDa.

DISCUSSION

DAG was discovered as a lipid second messenger through its involvement in PKC activation (31,32). At present there is increasing interest in the question as to whether DAG derived from PtdCho may function as a lipid second messenger and regulate PKC analogous to DAG derived from $PtdInsP_2$. Among the pathways that generate lipid second messengers from PtdCho, PtdCho-PLC is one of the least characterized. It has been reported that PtdCho-PLC is involved in the activation of the nuclear transcription factor κB in response to the tumor necrosis factor α (TNF-α) (33,34) as well as in the activation of the signal transducer and activator of transcription 6 (STAT6) induced by interleukin-4 (35). In immune responses and inflammatory processes, PtdCho-PLC may play important roles because it co-localizes with perforin-carrying granules and it is involved in natural killer (NK)-mediated lytic activity (36). In addition, PtdCho-PLC inhibition blocks interleukin-6 and TNF release in LPS-stimulated human alveolar macrophages (17). However, little is known about PtdCho-PLC implications in signal transduction pathways in CNS.

In this work we studied the existence of the PtdCho-PLC pathway in rat CC Syn. Tensioactive agents such as T X-100 and DOC stimulated synaptosomal DAG formation. Maximal DAG generation was observed at 0.1% T X-100, and DAG formation increased by 330% with respect to the control condition (Fig. 1). Our results agree with data regarding PtdCho-PLC activity in rat platelets (9). However, rat platelet PtdCho-PLC activity was highest at 0.01% DOC.

To corroborate the source of synaptosomal DAG generation, ethanol was used as a marker of PLD activity. It is known that PLD in the presence of primary alcohols, such as ethanol or 1-butanol, not only catalyzes PtdCho hydrolysis yielding PtdOH but also catalyzes a competitive transphosphatidylation reaction leading to the formation of either PtdEth or phosphatidylbutanol with the accompanying suppression of DAG formation from PtdOH (37,38). In the presence of 2% ethanol, DAG formation decreased by 27 and 42% at 5

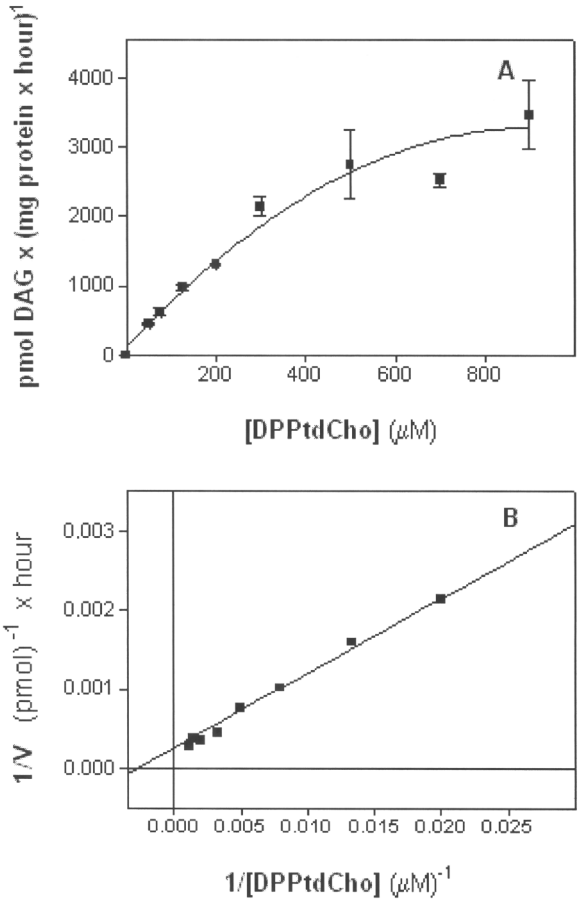

FIG. 5. (A) Determination of PtdCho-PLC K_m and V_{max}. Enzyme reactions were carried out in the presence of increasing concentrations of exogenous dipalmitoylphosphatidylcholine (DPPtdCho; 50, 75, 125, 200, 300, 500, 700, and 900 μM), 150,000 dpm of [^{14}C]DPPtdCho per assay in the presence of 2% ethanol, and 0.1% T X-100. Results are expressed as pmol DAG × (mg protein × h)$^{-1}$. (B) Lineweaver-Burk plot from the data in Figure 5A. Each experiment was performed twice, using a pool of three animals on each occasion. Data are the mean ± SD of four samples. For other abbreviations see Figure 1.

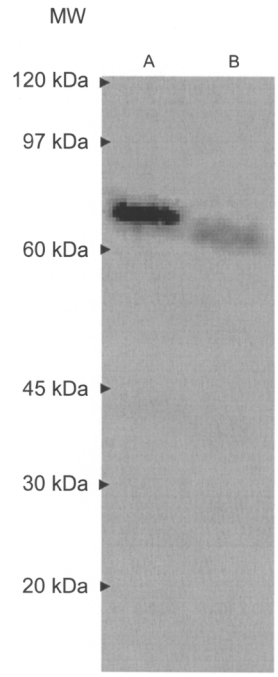

FIG. 6. Detection of synaptosomal PtdCho-PLC by Western blot assay. Synaptosomal proteins (60 μg) were resolved in a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was then incubated with rabbit polyclonal anti-PtdCho-PLC antibody (Ab), and immunoreactions were detected with polyclonal horseradish peroxidase conjugated to total antirabbit Ab. *Clostridium perfringens* PtdCho-PLC was used as a positive control for the anti-PtdCho-PLC Ab (A). Synaptosomal proteins from adult rats (4-mon-old) were loaded on the second line (B). For other abbreviations see Figure 1.

and 20 min, respectively (Fig. 2A). Consequently, the PtdCho-PLC contribution decreased from 73 to 58% as a time function. This decrease in DAG generation induced by ethanol demonstrates that PLD/PAP2 and PtdCho-PLC pathways differentially contribute to DAG production as a time function in CC synaptic endings (Fig. 2B). Our experiments suggest that PtdCho-PLC could be activated upstream from the PLD/PAP2 as it has been reported in other experimental systems (12,13,39).

For additional confirmation of the origin of DAG in CC Syn, enzyme assays were conducted in the presence of D609. This antiviral and antitumoral potassium xanthate is widely accepted as a selective inhibitor of PtdCho-PLC (15,40–42), and it has been proposed that it behaves as a competitive inhibitor of the enzyme (41,43). D609 inhibited synaptosomal DAG generation in a dose-dependent manner (Fig. 3). At 20 min of incubation, 500 μg/mL D609 decreased DAG formation by 61%, which was correlated with the PtdCho-PLC contribution observed in the presence of 2% ethanol.

Previous studies suggest that some of the biological events that had been attributed to PtdCho-PLC could be due to SMS (44,45). SMS is an enzyme involved in SM and ceramide metabolism that transfers a phosphocholine group from PtdCho to ceramide to generate SM and DAG (46,47). However, the kinetics of DAG production by SMS is significantly different from that of DAG production by PtdCho-PLC or PLD/PAP2 pathways because SMS-mediated DAG production is a slow process. Moreover, when [^{3}H-choline]DPPtdCho was used as a labeled substrate, no SM formation from PtdCho was detected under our experimental conditions (data not shown).

Experiments performed in the SPM and Sol fractions from Syn provide evidence that PtdCho-PLC is located in the plasma membrane (Fig. 4A). PtdCho-PLC localization in the membrane fraction is in accordance with previously published data observed in NK cells (36) and in NIH-3T3 fibroblasts (15). The inhibition of DAG generation by ethanol demonstrates that both PLD/PAP2 and PtdCho-PLC pathways contribute to DAG generation in SPM.

Although the molecular structure of eukaryotic PtdCho-PLC is still unknown, Clark *et al.* (48) demonstrated that antibodies prepared to *B. cereus* PtdCho-PLC cross-react with a

PtdCho-PLC from mammalian cells. Detection of synaptosomal PtdCho-PLC by Western blot assay with a polyclonal Ab raised against *B. cereus* PtdCho-PLC showed a band corresponding to 66 kDa (Fig. 6). Using also a polyclonal Ab directed to *B. cereus* PtdCho-PLC, a 66 kDa band was detected in NIH-3T3 fibroblasts (15) and in human NK cells (36). Our results also correlate to those obtained by Sheikhnejad and Srivastava (10), who determined the M.W. of an isolated PtdCho-PLC from bull seminal plasma by SDS-gel electrophoresis and reported two bands of 69 and 55 kDa.

Our experiments demonstrate the existence of a novel synaptosomal DAG generation pathway, thus confirming the first evidence for the presence of PtdCho-PLC in rat CC Syn. The existence of this novel lipid second messenger pathway in CNS is the starting point for the study of its involvement in neuronal signal transduction.

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Diacylglycerol Acyltransferase Activity and Triacylglycerol Synthesis in Germinating Castor Seed Cotyledons

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ABSTRACT: The central importance of storage lipid breakdown in providing carbon and energy during seed germination has been demonstrated by isolating the genes encoding the enzymes involved in FA β-oxidation. In contrast, little is known about the ability of germinating seeds to synthesize TAG. We report that castor cotyledons are capable of TAG synthesis. The rate of incorporation of ricinoleic acid into TAG reached a peak at 7 d after imbibition (DAI) (1.14 nmol/h/mg) and decreased rapidly thereafter, but was sustained at 20 DAI in cotyledons and true leaves. The castor DAG acyltransferase (RcDGAT) mRNA and protein were expressed throughout seed germination at levels considerably enhanced from that in the dormant seed, thus indicating new expression. Significant degradation of the RcDGAT protein was observed after 7 DAI. The DGAT activity was found to be predominantly a function of the level of the intact RcDGAT protein, with the rate of TAG synthesis decreasing as degradation of the RcDGAT protein proceeded. A possible mechanism for the degradation of the RcDGAT protein is discussed. The induction of DGAT mRNA and protein, the capacity for TAG synthesis *in vitro* and in tissue slices, and the differing TAG composition of dormant seed TAG vs. cotyledonary TAG provide strong circumstantial evidence for active TAG synthesis by cotyledons. However, we have not yet determined the physiological significance of this capability.

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Castor plants (*Ricinus communis*) are grown for their seeds, which, at maturity, contain up to 60% of oil. Nearly 90% of the TAG FA content is ricinoleic acid, a hydroxy FA that has numerous industrial uses (1). Unlike the majority of oil crops, castor seeds store oil in a living endosperm rather than the embryo. During castor seed germination, storage oil in the endosperm is converted to sucrose *via* successive pathways of β-oxidation, the glyoxylate cycle, the partial tricarboxylic acid cycle, and gluconeogenesis for utilization by the developing embryo (2).

Although the breakdown of storage lipids is the most striking metabolic event occurring during oilseed germination (3), it is evident from data presented in previous studies that germinating seeds may also be able to synthesize TAG. In 1975, Harwood reported that all the enzymes required in FA and glycerolipid synthesis were present and active in soybean cotyledons during seed germination (4). The synthesis of TAG has also been shown to occur during seed germination of some plants, such as the pea (5), cucumber (6), and soybean (7). These observations suggest the presence of DAG acyltransferase (DGAT) in germinating seeds. Recently, we identified a cDNA encoding DGAT from castor seed (RcDGAT) based on its homology to other plant-type DGAT1 cDNA (8) and investigated the expression of the RcDGAT gene and DGAT activity in developing seeds (9). In this study we extend our examination of the RcDGAT gene and protein to cotyledons of germinating castor to elucidate the function of RcDGAT in the germination process and in plant development.

EXPERIMENTAL PROCEDURES

Growth conditions. Castor (*Ricinus communis* L.) seeds, PI 215769, were obtained from the USDA Germplasm Resources Information Network, Southern Regional Plant Introduction Station (Griffin, GA). Plants were germinated and grown in the greenhouse at temperatures ranging between 28 (day) and 18°C (night), with supplemental metal halide lighting used to provide a 15-h day length (1000–1250 μeinstein/m^2/s). The time of planting was considered to be time zero in developmental studies.

Northern blot analysis. RNA samples were extracted from germinating castor bean cotyledons and true leaves using the method of Gu *et al.* (10). Five micrograms of total RNA was applied to each lane in a 1% agarose gel with 2% formaldehyde. The probe used for hybridization was generated by PCR labeling with digoxigenin (DIG-dUTP) using RcDGAT cDNA as the template and the primers: 5′-AAGACCCCATGGCGATTCTCGAAACGCCAGAA-3′ and 5′-CTGGAGCTTCAGAACCCTCTCAA-3′. Northern analysis was performed based on the DIG Application Manual for Filter Hybridization (Roche Molecular Biochemicals, Mannheim, Germany).

Microsomal preparations. Leaf samples were collected from developing seedlings. Microsomes were isolated as described by Lu *et al.* (11). Briefly, leaves were homogenized in buffer containing 400 mM sucrose, 100 mM Hepes-NaOH (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, and a protease inhibitor cocktail tablet per 10 mL (Boehringer Mannheim, Indianapolis, IN). The homogenate was cen-

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Abbreviations: DAI, days after imbibition; DGAT, diacylglycerol acyltransferase; RcDGAT, diacylglycerol acyltransferase from *Ricinus communis*.

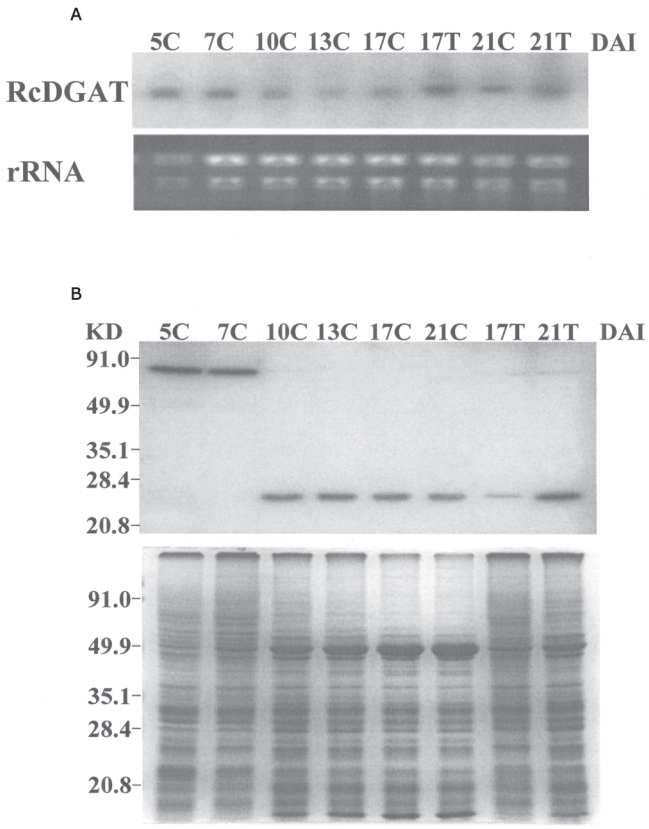

FIG. 1. Expression of castor DAG acyltransferase (RcDGAT) in germinating seedlings. RNA and protein samples were extracted from the cotyledon (C) or true leaves (T) at different stages of seed development from 5–21 d after imbibition (DAI). (A) Total RNA (5 μg) was loaded in each well of the gel for the northern blot analysis and hybridized to the RcDGAT cDNA probe. Ribosomal RNA (rRNA) was stained with ethidium bromide to show equal loading. (B) Total protein (30 μg) was used in SDS-PAGE. Top panel: The western blot was incubated with antibodies against peptide in the C-terminus of RcDGAT. Bottom panel: Duplicate protein gel stained with Coomassie Brilliant Blue R-250 is shown for equal loading of protein.

trifuged at 10,000 × *g* for 10 min to remove cell debris, and the supernatant was spun again at 100,000 × *g* for 90 min. The pellet was resuspended in the homogenizing buffer (microsomal fraction), and the protein concentration was determined using Bradford reagent (BioRad, Hercules, CA). These microsomal preparations were stored at −80°C and used for western blot and DGAT activity assay.

Western blot analysis. Thirty micrograms of microsomal protein from cotyledons and true leaves was separated by SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane. The membrane was incubated with the anti-RcDGAT antibodies developed previously (9) at 1:5000 dilution, followed by horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies (Amersham Pharmacia, Piscataway, NJ) at 1:3000 dilution. Horseradish peroxidase activity was visualized by chemiluminescence using the ECL kit (Amersham, Arlington Heights, IL).

In vitro DGAT assay. The DGAT assay was performed as in Cases *et al.* (12) with minor modifications. The [1-^{14}C]ricinoleoyl-CoA was synthesized according to McKeon *et al.* (13). Assay mixtures (100 μL) contained 0.1 M Tris-HCl (pH 7.0), 20% glycerol, 400 μM 1,2-diricinolein prepared previously (14), and 20 μM [^{14}C]ricinoleoyl-CoA (200,000 cpm). Reactions were started by the addition of 100 μg microsomal protein. The reactions were incubated for 15 min at 30°C with shaking and stopped by the addition of 1 μL 10% SDS. The 1,2-diricinolein was prepared as a 10 mM stock in 0.5% Tween-20. Lipids were extracted from assay mixtures using chloroform/methanol as previously described (15). The molecular species of TAG products were separated using C18 HPLC (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman Instruments Inc., Fullerton, CA) (16). DGAT activity was determined based on the ^{14}C-label incorporated into the TAG products from [^{14}C]ricinoleoyl-CoA.

Feeding cotyledons and true leaves with exogenous FA. Castor seedling leaves (0.15 g) were sliced into 1 × 5 mm^2 pieces and incubated at 25°C with gentle shaking for 2 h in 1 mL of 0.1 M sodium phosphate buffer (pH 7.2) containing FA (2 mM cold ricinoleic acid and 1.0 μCi [^{14}C]ricinoleic aicd). Assays were terminated by removing the incubation buffer and washing the samples three times with water. The lipids were extracted and analyzed as described below to measure the amount of label incorporated into TAG.

Lipid analysis. Lipids were extracted from 0.15 g of leaves at each developmental stage according to the method of Christie *et al.* (17). Samples were finally stored in methanol with 0.01% BHA at −20°C prior to HPLC analysis. Lipid classes and molecular species of TAG were separated by HPLC as we reported previously and identified by co-chromatography with a lipid standard, matching the retention times from the UV detector (absorbance at 205 nm) and flow scintillation analyzer (18).

RESULTS AND DISCUSSION

Expression of RcDGAT in leaves during castor bean germination. Northern analysis of RNA isolated from cotyledons and the first true leaves showed that RcDGAT was expressed at the transcriptional level in all samples tested (Fig. 1A). This result is in agreement with previous reports for *Arabidopsis* (11,19). In contrast, in immature castor seeds, the RcDGAT protein was not detectable until 1–2 wk after we were able to detect the RcDGAT mRNA. To determine whether the same transcription–translation "uncoupling" occurs in germinating seed, we performed western blot analysis of microsomal proteins from cotyledon and true leaf samples using antibodies against a 15-amino acid peptide in the C-terminus of RcDGAT (9). We detected a significant amount of RcDGAT protein in cotyledon samples at 5–7 d after imbibition (DAI), with most present as full-length protein (~60 kDa) as opposed to the partially degraded form prominent in developing seed (~50 kDa) (9). Previously, we found only trace amounts of mRNA and DGAT protein in the mature, dormant castor seed (9), so we conclude that their presence in the germinating cotyledon is a result of

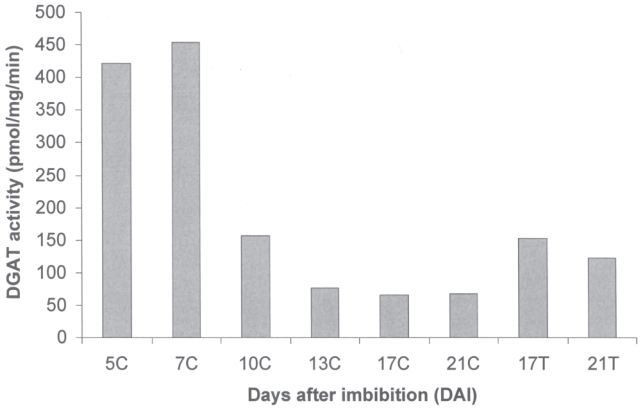

FIG. 2. DAG acyltransferase (DGAT) activities in the C and T from 5 to 21 DAI. Microsomal fractions were used for the DGAT assay. DGAT activities were measured based on the [^{14}C]-label incorporated into the TAG products. Data represent one of three independent experiments. For other abbreviations see Figure 1.

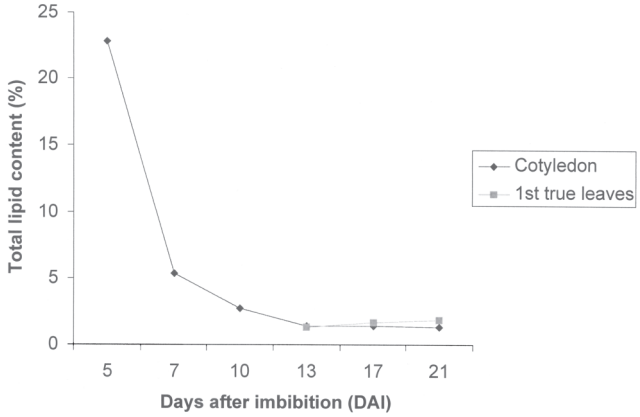

FIG. 3. Changes of lipid content in the cotyledon and true leaves during castor germination. Lipid content was measured as the percentage of total lipids to fresh weight. At each time point, 0.5 g of leaf sample was used to analyze for lipid content. For abbreviation see Figure 1.

new expression, not carryover. However, RcDGAT protein was rapidly degraded after 7 DAI. From 10 DAI, almost all RcDGAT protein in the cotyledons had degraded, whereas only small amounts remained intact in true leaf samples at 17 and 21 DAI (Fig. 1B). We do not know whether degradation of RcDGAT protein during seed germination reflects the *in vivo* situation or whether proteolysis occurred during preparation of the protein extract. However, adding protease inhibitors to the extraction buffer did not block protein degradation (data not shown).

Change of DGAT activity in germinating castor beans. To examine the effect of RcDGAT protein degradation during seed germination, we measured DGAT activity in an *in vitro* assay using microsomes isolated from cotyledons and true leaves (Fig. 2). The highest enzyme activities in cotyledons were detected at 5–7 DAI, the time at which most of the RcDGAT was still present as intact protein. Enzyme activities decreased significantly in cotyledons at 10 DAI and in true leaves at 17 and 21 DAI. In these samples, only small amounts of RcDGAT protein were intact, with most degraded to ~28 kDa. We found much lower enzyme activity in cotyledons at 13–21 DAI (about 6–7 times lower than the activity at 7 DAI), and at these times, almost all RcDGAT protein had been degraded based on western blot analysis (Fig. 1B). This indicates that DGAT activity corresponds directly to the amount of full-length RcDGAT protein. The level of full-length RcDGAT protein is likely to be regulated specifically by proteases in the seedlings since the total amount of protein produced did not differ significantly in cotyledons during seed germination (Fig. 1B).

Change of lipid content during seed germination. The germinating castor bean has a considerable supply of oil to provide energy for growth and development of the seedling. We investigated the lipid content of cotyledons during castor bean germination. The results are shown in Figure 3. We observed a dramatic decrease of the total lipid content at the early stages. The value then changed from 23% of the fresh weight at 5 DAI to 5% at 7 DAI, with a slower decline from 7–13 DAI. At 13 DAI, the lipid content was 1% and remained at this low level thereafter. The lipid contents in the first true leaves were relatively stable from their appearance at 13 (1.3%) to 21 DAI (1.8%). These results suggest that at the early stage of germination, mobilization of storage oil for transfer into the embryo dominates the metabolism in cotyledons, but beyond 13 DAI, the lipid content represents some combination of residual mobilized lipids and *de novo* synthesized lipids.

Rate of TAG synthesis in castor seedling leaves. We showed that the DGAT gene is expressed in seedling tissues. Although lipid biosynthesis is clearly important for membrane production, DGAT has generally been considered to implement a diversion of membrane lipid synthesis to oil production in seeds. Detection of DGAT activity suggests the possibility of *de novo* synthesis of TAG in cotyledons. To determine whether germinating castor bean leaves are capable of synthesizing TAG, we examined the incorporation of [^{14}C]ricinoleic acid into TAG

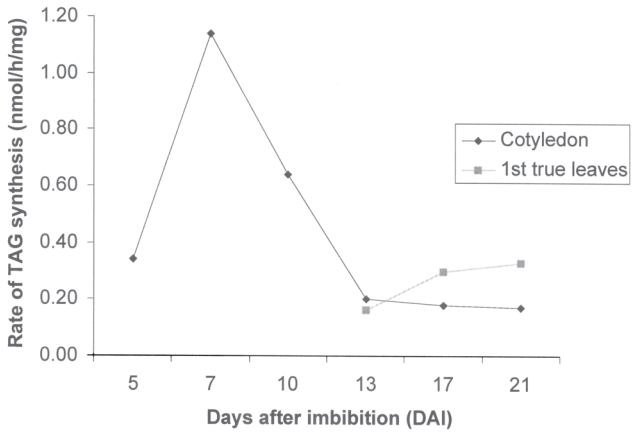

FIG. 4. Rate of ^{14}C-TAG synthesis in germinating castor seedlings. The cotyledon or true leaves (0.15 g) were sliced into small pieces and incubated with ^{14}C-labeled ricinoleic acid in 1 mL of 0.1M sodium phosphate buffer (pH 7.2) at 25°C for 2 h. The rate of ^{14}C-TAG synthesis was calculated as nanomoles of ^{14}C-ricinoleic acid found in TAG per hour in each milligram of tissue. Data represent one of three independent experiments. For abbreviation see Figure 1.

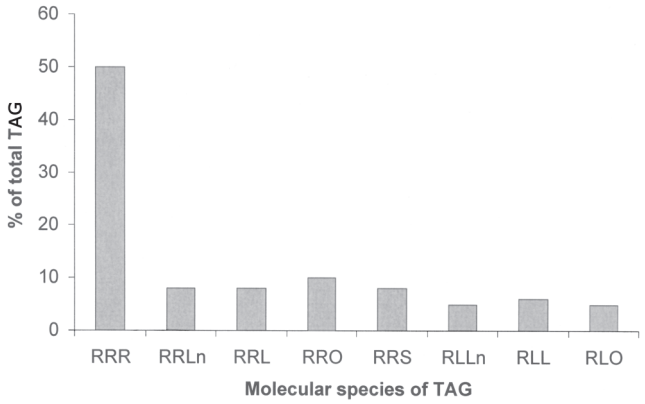

FIG. 5. Molecular species of TAG identified from castor cotyledons at 7 DAI and their contents. The data shown represent each molecular species of TAG as a percentage of total TAG. Abbreviations: R, ricinoleic acid; Ln, linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; for other abbreviations see Figure 1.

(Fig. 4). Sliced cotyledons or true leaves were incubated in buffer containing exogenous [^{14}C]ricinoleic acid. The rate of incorporation of ^{14}C from ricinoleic acid into TAG reached a peak at 7 DAI and rapidly declined after that. At 13 DAI, the incorporation rate was one-sixth that at 7 DAI and remained at a low rate to 21 DAI. We noticed that the flux of carbon from ricinoleic acid into TAG was slightly higher in growing true leaves than in senescing cotyledons after 13 DAI. To identify the representative molecular species of TAG that incorporated ricinoleic acid, a TAG fraction was collected from cotyledons at 7 DAI and separated by HPLC on a C18 column. We observed that about 50% of TAG labeled with ^{14}C was triricinolein, indicating that germinating castor cotyledons are not only capable of TAG synthesis, but also of producing oils with characteristics similar to mature castor (Fig. 5).

Castor beans store oil in a living endosperm, which is laterally attached to the cotyledons. The stored oil is mobilized from endosperm into the cotyledons during the first several days of germination. Over the same period, the activities of many enzymes involved in the pathway, including the β-oxidation pathway, the glyoxylate cycle, the tricarboxylic acid cycle, and gluconeogenesis, increase dramatically in the endosperm and the level of sugars (mainly sucrose) increases correspondingly (3). Huang and Beevers (20) reported that the accumulation of sucrose was much higher in endosperm lacking an embryo than with the embryo because in intact seedlings, sugars were absorbed by cotyledons, then used by the growing embryo at 5 DAI. This suggests that the major product of storage oil mobilization during germinative growth is sucrose. Our observations of lipid accumulation at 5 DAI showed that cotyledons contain about 23% lipids (Fig. 3), and 30–35% of that is TAG containing ricinoleate (data not shown). At 5 DAI, the oil reserves are depleted and the endosperm detaches from the cotyledons. The origin of the ricinoleic acid and TAG in the cotyledons is not clear. Since the FA composition differs from the stored oil, TAG is probably not transported directly from endosperms. Therefore, we tested the oleoyl-12-hydroxylase activity in the microsomal fraction of castor cotyledons at 5 DAI using [^{14}C]oleoyl-CoA as substrate (21). When using the method described previously (21), we detected a low level of oleoyl-12-desaturase activity but found no hydroxylase activity, even up to 0.5 mg of microsomal protein. This result suggests that the ricinoleic acid present in the cotyledon is directly mobilized from the endosperm. The appearance of radiolabel in TAG in the feeding experiment clearly indicated that during seed germination, cotyledons have the capacity for *de novo* synthesis of TAG, with the rate of TAG synthesis reaching a peak at 7 DAI and declining rapidly. From 13 DAI, cotyledons and true leaves maintained a basal level of TAG synthesis. These results are consistent with the change of RcDGAT protein and activity during germination (Fig. 1B, Fig. 2), except the rate of TAG synthesis at 5 DAI is much lower than that predicted based on the profile of the DGAT activity assay. One possible explanation for this discrepancy is that the FA content in the cotyledon at 5 DAI is much higher than at any other time point, and labeled exogenous FA could easily be diluted, resulting in an apparently low incorporation of [^{14}C] into TAG.

We have accumulated several different lines of evidence that we believe support the idea that castor cotyledons can carry out TAG biosynthesis. The induction of DGAT mRNA and protein, the capacity for TAG synthesis *in vitro* and in tissue slices, and the differing TAG composition of dormant seed TAG vs. cotyledonary TAG provide strong circumstantial evidence for active TAG synthesis by cotyledons. However, demonstration of such activity is not proof of physiological relevance, so we can only speculate about the underlying meaning of our observations of TAG synthesis in cotyledons from germinating seeds. We suggest that at early stages of germination, when plant photoautotrophism is not fully established, the TAG synthesized and stored in leaves provides a source of energy that maintains seedling development. During the germination of castor beans, storage fats in the endosperm are converted to sucrose with high efficiency, which can result in the accumulation of sugars. In seedlings grown on high levels of sucrose, levels of FA have been observed to be much higher than seedlings grown on low levels of sucrose (22). Since energy mobilization is of importance in early seedling development, it is possible that excess sugar production is channeled into TAG for storage or utilization to prevent osmotic effects. Three enzymes, i.e., DGAT1, DGAT2 (23), and PDAT (24), have been reported to have the ability to catalyze the final step of TAG synthesis. Whether DGAT2 and PDAT are involved in the TAG synthesis we observed in extracts from germinating seeds is not known, but we believe that RcDGAT is one of the rate-limiting enzymes for TAG synthesis in castor seed (9). Still, how this enzyme is regulated during the senescence of leaves is not at all clear. Figure 1B shows unambiguously that the RcDGAT protein is expressed throughout seedling development. A significant degradation of the RcDGAT protein is observed in cotyledons and true leaves after 10 DAI, and changes in enzyme activities closely reflect those of intact protein levels. If there is TAG biosynthesis during germination, then our data

suggest that TAG biosynthesis is regulated by the level of active RcDGAT protein, and proteolytic degradation of the protein regulates its activity. This is in contrast to DGAT expressed in castor seed development, during which the DGAT is partially degraded yet remains active. RcDGAT degradation may be associated with the accumulation and release of endopeptidases from ricinosomes during seed germination. In the castor bean, cysteine endopeptidase is a marker for ricinosomes (25), organelles found exclusively in plant tissues undergoing developmentally determined programmed cell death (26). The castor bean cotyledon represents a senescing tissue, programmed to die during germination after its cellular material has been mobilized for transfer into the embryo. The decline in measurable DGAT activity suggests that targeted proteolytic degradation may be one method for controlling DGAT activity in castor tissue.

ACKNOWLEDGMENTS

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Prediction of Adipose Tissue Composition Using Raman Spectroscopy: Average Properties and Individual Fatty Acids

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ABSTRACT: Raman spectroscopy has been used for the first time to predict the FA composition of unextracted adipose tissue of pork, beef, lamb, and chicken. It was found that the bulk unsaturation parameters could be predicted successfully [R^2 = 0.97, root mean square error of prediction (RMSEP) = 4.6% of 4 σ], with *cis* unsaturation, which accounted for the majority of the unsaturation, giving similar correlations. The combined abundance of all measured PUFA (≥2 double bonds per chain) was also well predicted with R^2 = 0.97 and RMSEP = 4.0% of 4 σ. *Trans* unsaturation was not as well modeled (R^2 = 0.52, RMSEP = 18% of 4 σ); this reduced prediction ability can be attributed to the low levels of *trans* FA found in adipose tissue (0.035 times the *cis* unsaturation level). For the individual FA, the average partial least squares (PLS) regression coefficient of the 18 most abundant FA (relative abundances ranging from 0.1 to 38.6% of the total FA content) was R^2 = 0.73; the average RMSEP = 11.9% of 4 σ. Regression coefficients and prediction errors for the five most abundant FA were all better than the average value (in some cases as low as RMSEP = 4.7% of 4 σ). Cross-correlation between the abundances of the minor FA and more abundant acids could be determined by principal component analysis methods, and the resulting groups of correlated compounds were also well-predicted using PLS. The accuracy of the prediction of individual FA was at least as good as other spectroscopic methods, and the extremely straightforward sampling method meant that very rapid analysis of samples at ambient temperature was easily achieved. This work shows that Raman profiling of hundreds of samples per day is easily achievable with an automated sampling system.

Paper no. L9812 in *Lipids 41*, 287–294 (March 2006).

Government dietary guidelines in the United Kingdom (UK) are based on the assumption that nutritional or dietary management, and in particular management of dietary lipid intake, can help reduce mortality and morbidity from cardiovascular disease (CVD). At present, UK Department of Health guidelines (1994) recommend that the contribution of lipid intake to total dietary energy should be reduced from about 40 to 30% of dietary energy intake and that the contribution from saturated FA in particular should be reduced from 15 to 10% of total energy intake (1). They also recommend that the ratio of PUFA to saturated FA in the diet should be increased (1). Many studies have identified the involvement of a high intake of saturated fat with increased risk of CVD (2–4), and specific saturated FA are now recognized to have hypercholesterolemic effects. In a review of studies investigating the cholesterolemic effects of individual FA, Kris-Etherton and Yu (5) concluded that the saturated FA 12:0–16:0, were hypercholesterolemic but that the unsaturated FA were hypocholesterolemic, with PUFA being more potent than monounsaturated FA. The essential n-3 FA, such as 18:3 α-linolenic acid, have also been demonstrated to have health benefits by lowering blood lipid concentrations and reducing the risk of platelet aggregation and thrombosis (6). Finally, in recent years there has been much interest and research on the potential health benefits of consumption of CLA, and the anticarcinogenic and antiatherosclerotic properties of some isomers of CLA have been demonstrated in animal studies and models (7–10).

The net result of all the foregoing is that it is becoming increasingly important to measure the abundance of individual FA in the complex mixtures of lipids that are found in foods. This is particularly true for samples that have been deliberately treated to move their FA composition away from "typical" values. For example, the saturated nature of animal lipids has adversely influenced their perception by consumers; this has led to research aimed at reducing the high level of saturated FA and increasing the proportion of PUFA in both meat and milk lipids through dietary manipulation (11–14).

This paper is part of a series that investigate the feasibility of applying Raman spectroscopy to the analysis of FA-based fats and oils (15–18). In previous experimental studies, the correlation between particular bands in the Raman spectra of solid and liquid samples of pure FAME (15) and properties such as average chain length was investigated. This experimental work on FAME was complemented by density functional calculations, which allowed the underlying reasons for the experimentally determined structure/spectra correlations to be explored (16,17). Application of the same approach to real foodstuffs (clarified butterfat) showed that even with mixed TG, rather than simple model FAME, bulk composition parameters, e.g., chain length or iodine value, could be determined from Raman spectra (18). Furthermore, in addition to bulk or average properties, it was also possible to build multivariate calibration models that allowed the abundance of individual FA to be determined (18). The ability to monitor levels of desirable/undesirable components is obviously important in dietary lipids.

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Abbreviations: CVD, cardiovascular disease; GC, gas chromatograph/chromatography; PCA, principal component analysis; PLS, partial least squares; RMSEP, root mean square error of prediction; UK, United Kingdom.

Here we extend the work on butterfat to investigation of adipose tissue; the aim is to find whether Raman spectra can still be used to predict FA composition when the samples are drawn from a much broader range of sources, including tissue from different species.

EXPERIMENTAL PROCEDURES

Samples. The samples used in this investigation were subcutaneous adipose tissue dissected from above the *Longisimus dorsi* in the position of the 12th rib for beef, lamb, and pork, and from above the breast for chicken. As it is well known that the FA composition varies between the inner and outer surfaces of adipose tissues, care was taken when dissecting the samples to note the outer (next to skin) and inner (next to muscle) surfaces of the adipose tissue. Samples were obtained from a number of research sources and were all dissected within 48 h of slaughter.

GC. GC was the primary analysis method in this study. Subsamples (*ca.* 0.2–0.5 g) of adipose tissue were dissected from the regions, on each surface of the samples, that had been Raman probed. These samples were then extracted three times in 10 mL of 2:1 chloroform/methanol. FAME were prepared from the extracted fat according to the British Standard method (British Standards Institute BS 684-2.34) and were analyzed by GC (18). Aliquots of FAME in heptane (0.1 μL) were injected *via* a septa programmable injector at 250°C onto a GC column (WCOT CP Sil-88; i.d. 0.25 mm; length 50 m; film thickness 0.2 μm) supplied by Varian Inc. (Middelburg, The Netherlands) fitted in a Varian 3400 gas chromatograph (GC). An FID was used, and the heating program was ramped as follows to improve separation of the FA peaks: 50°C for 1.0 min, 50 to 140°C at 20°C min^{-1}, 140 to 225°C at 5°C min^{-1}, and finally 225°C for 10.0 min. Pure FAME, obtained from Sigma-Aldrich (Poole, UK), were used to prepare internal and external standards to establish retention times and peak identification. The following acids, as well as all FA 20 carbons in length, were undetectable in at least one species: 12:0, 14:1*c*Δ9, 15:1*c*Δ10, 17:1*c*Δ10, and 18:2*t*Δ9,12. Only FA detectable in more than half the samples were investigated. The total combined abundance of PUFA was determined by summing the relative abundances of all the PUFA including those only detected in pork and chicken (half of the samples).

Raman spectroscopy. Raman measurements were carried out using a 785 nm excitation wavelength (typically 100–120 mW at the sample, 10 cm^{-1} resolution, 180° geometry) generated by a home-built spectrometer previously described (19). Raman spectra of 8–10 samples of each of the four species were recorded. The sample was mounted on a rotating stage, oriented with the surface perpendicular to the incident radiation. The laser was line focused on the surface of the sample while the stage was rotated, giving an approximate coverage of 1.5 cm^2. Spectra were accumulated from both sides (outer and inner) to give two separate spectra for each sample, yielding a total of 16–20 spectra per species. Wavelength calibration was carried out using a neon emission lamp. The spectrograph was calibrated for Raman shift using a 50:50 (vol/vol) acetonitrile/toluene mixture [comparing to frequency standards from the American Standard Testing Method (ASTM E 1840 (1996)]. The Raman signal was recorded from 270 to 1900 cm^{-1}, the region containing the C–C, C=C, C–O and C=O, stretches and the C–H bends, in two sequential 60-s accumulations.

The non-Raman background in the raw data was removed, and the spectra were normalized and mean-centered as previously described (18). Standard partial least squares (PLS) analysis was carried out between the Raman data and FA profiles (using PLS2 to simultaneously model the full range of FA) using The Unscrambler™ v9.1 (Camo, Trondheim, Norway) and leave-one-out cross-validation to confirm the predictive ability of the model generated. During the PLS analysis of the data the Uncertainty Test was used to select the wavenumber shifts correlated with the measured parameter and reject wavenumber shifts not contributing to the prediction. To test the predictive ability of the models on unknown samples, calibrations were generated using PLS2 with test set validation. The data set was prepared by removing a subset of 10 randomly selected spectra and the model constructed from the remaining 63 samples and validated using the subset of 10. All species were represented in the test set.

RESULTS AND DISCUSSION

Figure 1 shows the average Raman spectrum of adipose tissue from each of the species used in this experiment. The major bands have been assigned previously, but for convenience they are numbered on the Figure and the most important assignments are given in Table 1. The differences between the average adipose tissue spectrum of each species, as shown in Figure 1, are larger than the differences between the butterfats obtained under various feeding regimes that were studied previously (18). The average FA profiles of the four species studied and the corresponding average bulk properties are given in Tables 2 and 3, respectively. The data in Figure 1 show that the samples are partly melted because the spectra show features similar to those characteristic of both liquid FAME (which give only broad unresolved features in several spectral regions) and solid FAME (which typically display sets of narrow, well-defined bands (15). The differences between solid and liquid FAME are particularly noticeable at *ca.* 1000–1200 cm^{-1}, and in the spectra shown in Figure 1 there are sharp solid-phase peaks on top of underlying broad liquid-phase bands in this spectral range.

Since the Raman spectra contain readily identifiable bands associated with particular groups, such as C=C bonds or CH_2 groups, it would be expected that the Raman spectrum of a mixed sample, being an ensemble average of the spectra of the individual FA chains present, would reflect the bulk sample properties. For example, the determination of unsaturation (measured as C=C per chain or per gram, i.e., in molar or molal units, respectively) is the most widely investigated aspect of the Raman spectra of lipids. In the adipose tissue samples investigated here, the level of unsaturation (the number of *cis* un-

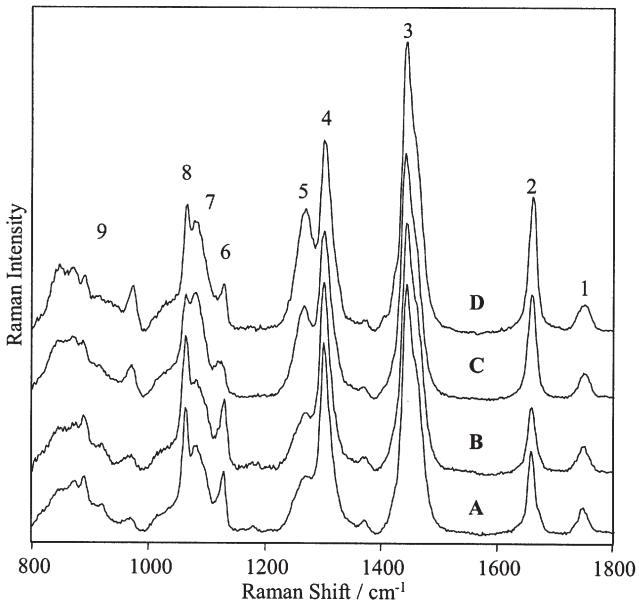

FIG. 1. Average Raman spectra for each species: (A) beef, (B) lamb, (C) chicken, and (D) pork. The numbering of the bands refers to the band positions and assignments given in Table 1. Each average spectrum has been normalized about the carbonyl stretch band at *ca.* 1750 cm^{-1}. Spectra acquired at 19–21°C.

saturated bonds per chain, i.e., molar unsaturation) was well-predicted using standard PLS2 methods, which gave a correlation coefficient of $R^2 = 0.97$ and a root mean square error of prediction (RMSEP) of 4.6% of the range. In contrast, it was not possible to find a good prediction model for *trans* unsaturation; the best model gave $R^2 = 0.52$ and RMSEP = 18% of the range. This poor *trans* prediction ability is due to the low range of relative abundances of *trans* unsaturated bonds in the adipose tissue (0.031, compared with 0.24 bonds per FA for *cis* unsaturated bonds). However, it has previously been demonstrated that Raman spectroscopy is capable of accurately predicting *trans* content in samples with a larger range of *trans* values (20). Total unsaturation (sum of *cis* and *trans*) was predicted with similar accuracy to the *cis* content, primarily because the low *trans* unsaturation meant that it was the well-predicted *cis* contribution that dominated total unsaturation.

In addition to unsaturation, another major compositional parameter is the average chain length. It was found that, as was the case for butterfats and model FAME, the Raman spectra could be used to predict average chain length satisfactorily, $R^2 = 0.92$ and RMSEP = 6.9% of the range. Since the chain length is the sum of the number of saturated and unsaturated C–C bonds and both chain length and unsaturation were modeled satisfactorily in this sample set, it would be expected that the third parameter, number of saturated C–C bonds, could also be modeled. However, it was found that even the best model gave an unsatisfactory correlation, $R^2 = 0.58$, for the average number of C–C bonds per chain. Although this result appears anomalous, it is a straightforward consequence of the fact that the number of saturated bonds per chain varied very little between samples ($\sigma = 0.07$, compared with 0.24 for unsaturated bonds), which made it difficult to model. In effect, the number of saturated bonds was almost constant in all the tissue samples so that modeling the increase in double bonds effectively also measured the increase in chain length over a constant baseline value of *ca.* 15.5 saturated C–C bonds per chain. In sample sets with a larger range of saturated C–C values it should be possible to obtain a better calibration.

The ability to predict bulk composition parameters from Raman spectra is not surprising if the spectra of individual FA are simply regarded as the sum of the contributions from individual entities, because incremental structural changes would be expected to give incremental increases in the intensities of the associated vibrational bands. For example, addition of CH_2 groups into the chain would be expected to increase the intensities of the vibrations associated with the methylene groups, such as the CH_2 twist and scissoring bands. However, if this model were strictly accurate, it would be impossible to determine relative proportions of the individual constituents in samples containing mixtures of different FA since there would be many ways of preparing different mixtures with the same bulk properties, which would therefore give the same overall spectrum. However, experimental and theoretical studies on model FAME showed that, whereas many of the vibrational modes did show the expected incremental changes with changing structure, there were some bands that did not follow a smooth progression and potentially might allow the variation in indi-

TABLE 1
Assignment of Bands in the Raman Spectra of Adipose Tissue at Ambient Temperature (19–21°C)

Band[a]	Band position (cm^{-1})	Assignment (ref.)
1	1730–1750	ν (C=O) Carbonyl stretch (27)
2	1670–1680	*trans* ν (C=C) Olefinic stretch (28)
	1650–1660	*cis* ν (C=C) Olefinic stretch (28)
3	1400–1500	$\delta\,(CH_2)_{sc}$ Methylene scissor deformations (27)
4	1295–1305	$\delta\,(CH_2)_{tw}$ Methylene twisting deformations (29)
5	1250–1280	$\delta\,(=CH)_{ip}$ In-plane *cis* olefinic hydrogen bend (29)
6	1100–1135	$\nu\,(C–C)_{ip}$ In-phase aliphatic C–C stretch *all trans* (30)
7	1080–1090	$\nu\,(C–C)_{g}$ Liquid: aliphatic C–C stretch in *gauche* (31)
8	1060–1065	$\nu\,(C–C)_{op}$ Out-of-phase aliphatic C–C stretch all-*trans* (32)
9	800–920	$\nu\,(C_1–C_2)$, $CH_{3\ rk}$, ν (C–O) Complex broad table in liquid (33)

[a]The band number refers to the numbering in Figure 1.

TABLE 2
Average FA Profile of Each of the Animal Species Used to Prepare the Calibration for the Raman Spectra

FA	Lamb[a]	Beef[a]	Pork[a]	Chicken[a]
14:0	4.18	4.09	1.16	0.79
14:1*c*Δ9	1.26	1.47	ND[a]	0.19
15:0	0.65	1.20	ND	0.12
16:0	28.83	28.74	20.46	23.23
16:1*c*Δ9	5.00	5.67	1.67	5.80
17:0	1.08	1.17	0.33	0.12
17:1*c*Δ10	0.95	ND	0.22	0.25
18:0	13.91	11.95	10.39	5.47
18:1*c*Δ9	37.53	39.37	32.70	45.05
18:1*t*[b]	2.53	1.97	0.09	0.78
18:2*c*Δ9,12	1.03	2.18	26.55	14.90
18:2*t*Δ9,12	0.26	2.19	ND	ND
18:3*c*Δ6,9,12	0.19	ND	0.09	0.27
18:3*c*Δ9,12,15	0.57	ND	2.60	1.99
20:0	0.21	ND	0.16	0.02
20:xc[c]	1.58	ND	3.47	0.88

[a]Values quoted are g/100 g FA. ND = not detected.
[b]The isomers for 18:1*t* are not well resolved, so the figure is for an undifferentiated mixture of isomers.
[c]The various 20-carbon unsaturated FA could not be unambiguously assigned under experimental conditions, and so have been grouped together.

TABLE 3
The Average Chemical Subunit Content[a] for Each of the Species, Derived from the Subset Used for GC Analysis

Parameter (per chain)	Species	Average	SD
Total number of Carbons (no. of C)	Beef	16.576	0.044
	Pork	16.375	0.077
	Chicken	16.117	0.029
	Lamb	16.024	0.055
Total number of saturated bonds (no. C–C)	Beef	15.599	0.042
	Pork	15.528	0.017
	Chicken	15.488	0.007
	Lamb	15.436	0.037
Total number of unsaturated bonds (no. C=C)	Pork	1.030	0.076
	Chicken	0.903	0.020
	Beef	0.572	0.038
	Lamb	0.548	0.059
trans:cis Ratio (%)	Beef	13.341	7.818
	Lamb	6.080	2.019
	Chicken	0.872	0.096
	Pork	0.089	0.049

[a]For each chemical subunit the samples are arranged in descending order.

TABLE 4
Regression Coefficients and Standard Errors of Prediction for the Prediction of FA Content and Bulk Parameters Using Raman Spectra

				RMSEP[d]				No.	No.
	Mean[a]	σ[b]	R^2 [c]	Absolute[d]	(% Mean)[d]	(% 4 σ)[d]	RMSEE[e]	samples	Factors
14:0	2.55	1.66	0.793	0.751	29.5	11.3	0.494	72	4
14:1*c*Δ9	0.71	0.74	0.472	0.517	72.7	17.5	0.416	53	4
15:0	0.5	0.48	0.823	0.202	40.3	10.5	0.063	72	4
16:0	25.3	4.08	0.892	1.304	5.2	8.0	1.362	72	1
16:1*c*Δ9	4.51	2.03	0.834	0.804	17.8	9.9	0.684	72	3
17:0	0.68	0.5	0.819	0.21	30.8	10.5	0.150	72	3
17:1*c*Δ10	0.47	0.36	0.7	0.206	43.9	14.3	0.054	54	5
18:0	10.46	3.78	0.89	1.257	12	8.3	1.403	72	3
18:1*c*Δ9	38.61	5.48	0.796	2.476	6.4	11.3	1.842	72	2
18:1*t*[f]	1.371	1.56	0.752	0.81	59.1	13.0	0.843	63	4
18:2*c*Δ9,12	11.18	10.99	0.965	2.058	18.4	4.7	2.278	72	3
18:2*t*Δ9,12	1.25	1.13	0.613	0.719	57.5	15.9	0.597	38	3
18:3*c*Δ9,12,15	1.71	0.93	0.932	0.284	16.6	7.6	0.205	54	3
18:3*c*Δ6,9,12	0.18	0.09	0.593	0.053	29.7	14.7	0.030	54	6
20:0	0.13	0.1	0.719	0.055	42.5	13.8	0.029	54	4
20-Carbon unsaturated[g]	1.977	1.190	0.890	0.392	19.8	8.2	0.45	54	5
Chain length	17.3	0.23	0.922	0.063	0.4	6.9	0.054	73	3
Saturation	15.51	0.07	0.575	0.046	0.3	16.3	0.037	73	3
Unsaturation	0.76	0.22	0.965	0.041	5.4	4.6	0.033	73	3
cis Unsaturation	0.73	0.24	0.965	0.045	6.2	4.7	0.041	73	3
trans Unsaturation	0.026	0.031	0.516	0.022	82.7	17.9	0.020	73	3
cis/trans	5.1	6.57	0.499	4.66	91.4	17.7	3.81	73	8
PUFA[h]	17.3	12.64	0.973	2.01	11.6	4.0	2.54	56	3
Group 1	12.46	34.8	0.971	2.07	16.6	5.9	2.32	73	3
Group 2	42.94	28.4	0.861	2.70	6.3	9.5	3.76	73	7
Group 3	6.35	12.6	0.926	2.00	31.6	15.8	1.96	73	2
Group 4	1.27	3.4	0.925	0.30	23.8	9.0	0.42	73	7
Group 5	10.77	18.0	0.884	1.31	12.2	7.3	1.62	73	3

[a]Mean weight percentage of total FA (g/100 g FA) for all samples used within investigation.
[b]SD of the % total mass of lipid from the mean.
[c]Least squares regression correlation coefficient (validation).
[d]Root Mean SE of prediction (RMSEP) as absolute value and as percentage of mean (RMSEP % of μ) and sample range (RMSEP % of 4 σ).
[e]Root Mean SE of estimate (RMSEE), determined by test set validation, removing a subset of 10 samples.
[f]The isomers for 18:1*t* are not well resolved, so the figure is for an undifferentiated mixture of isomers.
[g]These FA are unassigned due to difficulty in unambiguously identifying the different 20-carbon unsaturated FA under the experimental conditions used in this study.
[h]PUFA represents the combined abundances of all measured FA with ≥2double bonds in the chain. FA groups, as determined by grouping in principal components analysis: Group 1 = 18:3*c*Δ9,12,15; 18:2*c*Δ9,12; 20:xa ; Group 2 = 18:2*t*Δ9,12; 18:1*c*Δ9, 18:3Δ6,9,12; 16:1*c*Δ9; Group 3 = 16:0, 18:1*t*Δ9; 15:0, 17:1*c*Δ10; 14:0; 14:1*c*Δ9; 17:0; Group 4 = 20:xb; 20:xc*; Group 5 = 20:0, 18:0. *Here the symbols 20xa–c stand for three different 20-carbon FA, which could be distinguished in the GC but could not be unambiguously identified.

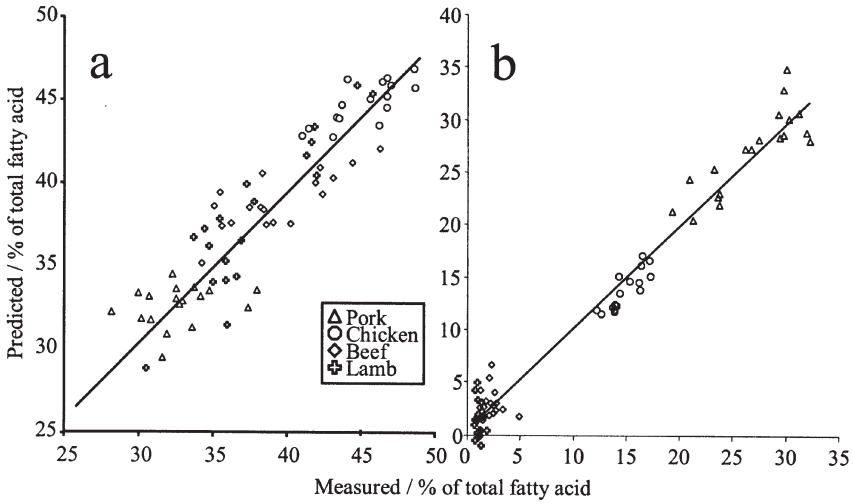

FIG. 2. Partial least squares (PLS) regression validation plot for (a) percentage of 18:1*c*Δ9 and (b) percentage of 18:2*c*Δ9,12 compared with total FA content (g/100 g FA) in subcutaneous adipose tissue predicted from Raman spectra against the percentage of total FA measured by GC.

vidual constituents to be determined, even in mixed samples with the same bulk composition (15,17).

Previously it was found that PLS1 regressions of Raman data against the amounts of individual FA in butterfat samples (18) (determined by GC as percentage of total FA) could be used to predict the relative proportions of the major FA in butterfat. In the previous study PLS1 regressions were used to create individual calibration models for each FA; in this study PLS2 was used so that we could generate a single model that predicted all the FA in the profile. The PLS2 approach gives slightly reduced prediction accuracy but it is preferable owing to its inherent simplicity. Table 4 shows the results for a PLS2 regression carried out for 73 samples of subcutaneous adipose tissue. The samples were taken from four different species, which ensured the extent of variation of each individual FA was acceptably large, but they were not separated according to

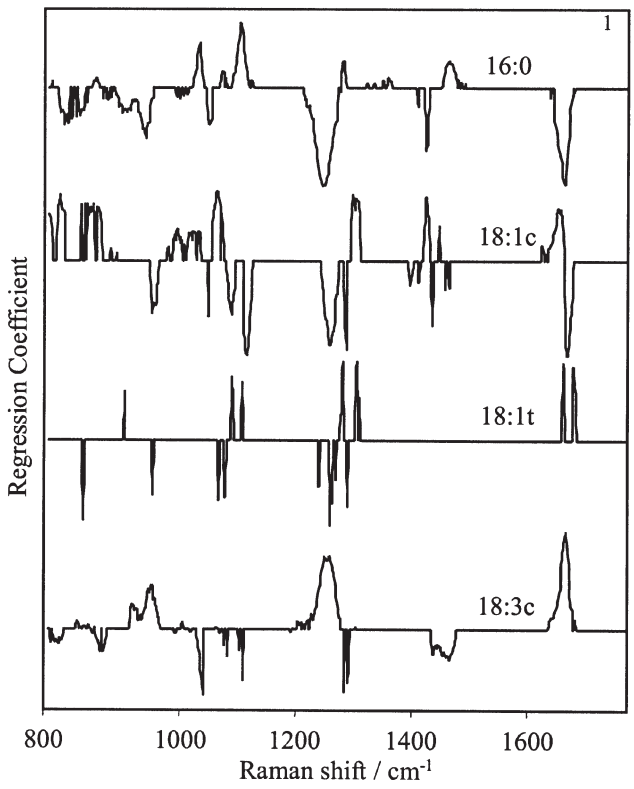

FIG. 3. PLS regression coefficients used to predict, from the Raman spectra of intact tissue at 19–21°C, the percentage of total FA content of 16:0, 18:1*c*Δ9, 18:1*t*, and 18:3*c*Δ9,12,15 in subcutaneous adipose tissue, as determined by GC. For abbreviation see Figure 2.

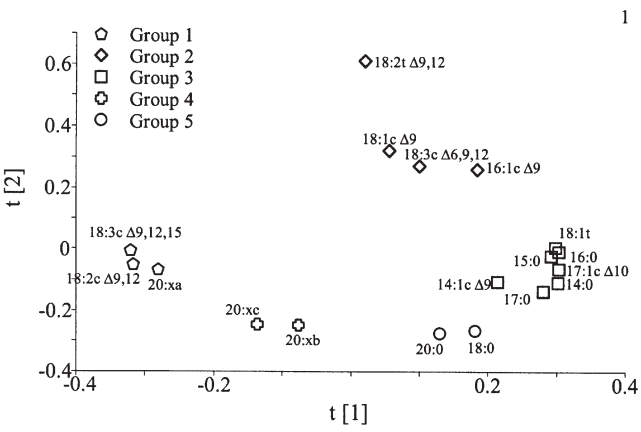

FIG. 4. Principal components analysis loading plot for the GC data, showing the groups of interrelated FA present in adipose tissue. Group 1 = 18:3*c*Δ9,12,15, 18:2*c*Δ9,12,20xa; Group 2 = 18:2*t*, 18:1*c*Δ9, 18:3*c*Δ6,9,12, 12:0, 16:1*c*Δ9; Group 3 = 16:0, 18:1*t*, 15:0, 17:1*c*Δ10, 14:0, 14:1*c*Δ9, 17:0; Group 4 = 20:xb 20:xc; Group 5 = 20:0, 18:0. Here the symbols 20xa–c stand for three different 20-carbon FA, which could be distinguished in the GC but could not be unambiguously identified.

species for the purpose of regression. The Table shows that the four most abundant FA analyzed had regression correlation coefficients ranging from R^2 = 0.80 to 0.97, with standard errors of prediction ranging from 4.7 to 11.3% of the range (4 σ). Example regression plots of the most common FA (18:1*c*Δ9) and that of the FA with the largest range (18:2*c*Δ9,12) are shown in Figure 2. Of all the moderately abundant (>3%) FA, 18:1*c*Δ9 gave the poorest correlation (due to the relatively narrow range of values, range = 14% of the mean, compared with 98% of the mean for 18:2*c*Δ9,12) but it is clear from Figure 2 that even in this worst case the scatter in the plot is still reasonably small with no systematic error apparent. This is reflected in the prediction error of 6.4% of the mean, which is acceptably low for a rapid, nondestructive analytical method. These FA are of particular interest as they confer the predominant chemical and physical characteristics of the lipids within the adipose tissue and furthermore have important consequences in health and nutrition. For example, 16:0 is known to be hypercholesterolemic, whereas the *cis* unsaturated acids lower cholesterol (in particular, LDL).

The loadings used to predict the proportion of selected FA from the Raman spectrum are presented in Figure 3. The pattern of bands used to predict each FA agrees well with well-established assignments of the bands in the Raman spectrum of FAME. The saturated FA 16:0 has positive correlation coefficients in positions known to arise from saturated modes (e.g., 1440, 1300, 1130, and 1060 cm^{-1}) whereas bands related to unsaturated modes are negative (e.g., 1270 and 1660 cm^{-1}). Conversely, the unsaturated FA would be expected to have positive coefficients for unsaturated modes and negative coefficients for saturated modes. The weighting plot for 18:3*c*Δ9,12,15 shows the expected pattern, such as large positive bands at 1660 (*cis* C=C stretch) and 1270 cm^{-1}. The 18:1*t* FA shows some of the expected bands such as positive bands at the *trans* C=C stretch position (1670–1680 cm^{-1}), but the correlation is less obvious than the 18:3*c*Δ9,12,15, which is hardly surprising given the low abundance in the sample and the corresponding high prediction error. The weighting plot for 18:1*c*Δ9 is complicated by the occurrence of strong negative and positive components, which, although they presumably arise from changes in saturation from the mean values, are so close to those values (18:1*c* is the predominant FA in adipose samples) that the model can only find a very complex relationship between the abundance of 18:1*c* and the spectra.

Surprisingly good predictions were obtained for a number of the less abundant FA, with 15:0 and 17:0 giving R^2 = 0.82 and RMSEP = 10.5% of the range, despite being present in relative abundances of 0.5% of the total FA content. It seems highly unlikely that these low-abundance compounds give distinct characteristic signals that are detectable under our experimental conditions, since the intensity of even the strongest bands in a FA present at <1% would be similar to the noise in the data, and the differences that would allow the particular FA to be distinguished from similar compounds in the tissue would be expected to be much smaller again. A more convincing explanation is that this good modeling ability arises from an underlying correlation between the low-abundance FA and much more abundant ones whose signals would be expected to be detectable. Since the adipose tissue is a product of biosynthetic pathways, such cross-correlation might be expected. In this study the samples were dissected from four animal species (two ruminant and two nonruminant) with a selection of breeds from within each species to minimize cross-correlation. Moreover, a range of experimental programs and commercial suppliers was used to reduce the influence of feeding regimes. The consequence is that the cross-correlation is significantly less than that in the butterfat samples previously studied (18), so the correlation determined within this sample set is more widely applicable.

One way of explicitly including the cross-correlation in the analysis and simplifying the data is to carry out a principal components analysis (PCA) and divide all the FA into groups according to which others their abundance correlates with. Figure 4 shows such a plot for the FA in this study. The FA abundances for each of the five groups found in the PCA analysis of the data were then summed, and the Raman spectra were used to model the variation between the FA groups. The results of these regressions of the FA groups are summarized in Table 4, with details of contributing FA given in the footnote. The one obvious group is the FA set comprising 18:2*t*Δ9,12; 18:1*c*Δ9; 18:3Δ6,9,12; 12:0; and 16:1*c*Δ9 while another large group contains highly *cis* unsaturated compounds 18:2*c*Δ9,12, 18:3*c*Δ9,12,15, and 20xa (which is one of the unassigned 20-carbon unsaturated FA). The Raman spectra were able to predict the total abundance of all of these groups with some success. In fact, the least well-modeled group is the one constituting the largest fraction of the sample, although this may be attributed to the lower range of values relative to the mean abundance (range < 0.66 σ) that this group shows (in the remaining groups the range was >1.5 σ).

A further useful group that can be defined, though not cross-correlated by relative abundance, is the combined proportion of PUFA. This group of FA, as detailed above, is of extreme interest because of their relevance to health issues. Raman spectroscopy was able to model this group well (R^2 = 0.97, RMSEP = 4.0% of 4 σ). One individual FA that is of particular interest is the EFA 18:3*c*Δ9,12,15, which was well modeled (R^2 = 0.932) with good prediction accuracy (7.6% of 4 σ). Using a PLS1 regression allows models to be optimized for any given FA. In this case, using PLS1 regression to predict 18:3*c*Δ9,12,15 gave a slightly better result than PLS2 (R^2 = 0.946, RMSEP = 5.8% of 4 σ). As already alluded to, Raman spectroscopy is able to predict the relative abundances of several FA that influence the level of cholesterol in the blood including 14:0 and 16:0, which increase cholesterol, and the unsaturated FA, which are important in reducing cholesterol. Unfortunately the limitations of the GC method used did not allow calculation of the cholesterolemic index, but the results suggest that Raman could be used to predict this important parameter. One FA of interest, CLA, was not detected in large quantities in concentrate-fed ruminants, most probably because it was not sufficiently resolved from the more abundant 18:3Δ9,12,15

on the GC column used in this study. However, it has been previously demonstrated that Raman spectroscopy can be used to predict the relative abundance of CLA in butterfats from grass-fed cows, where higher concentrations are found (18).

Overall, the prediction ability for the FA in this study on adipose tissue (average R^2 = 0.74 and average RMSEP = 12.0% of 4 σ for the 18 most abundant FA) was significantly better than was found in experiments carried out on clarified butter samples analyzed at 55°C [average R^2 = 0.68, and average RMSEP = 18.5% of 4 σ for the 18 most abundant FA (18)]. This improvement is not due to higher signal-to-noise ratios in the adipose spectra but seems to stem from the fact that the spectra of the adipose tissue were recorded at temperatures within the melting range whereas those of the butterfat were recorded on fully melted samples (although the slightly larger range of abundances for the FA in the adipose samples may also have contributed). Since the Raman spectrum of each FA changes with physical state, recording spectra in a temperature range that allows the adoption of different physical states, i.e., within the melting range, gives information that is simply lost at temperatures where all the components have melted. In this case the adipose tissue was run at 21 ± 2°C (ambient temperature), which allowed the higher m.p. TG to solidify and give rise to characteristic sharp bands that can easily be distinguished from the broader bands of the liquid constituents (shorter-chain or unsaturated components).

The results shown in Table 4 compare favorably with other published investigations that used spectroscopic techniques to predict FA composition. A number of papers investigating the use of NIR spectroscopy to predict FA composition of foodstuffs (21–24) found that it is possible to predict the proportion of the major unsaturated FA well (R^2 typically ≥0.95 for the most abundant unsaturated FA) but that the prediction ability is reduced as the proportion of the FA decreases and the saturation increases. Velasco *et al.* (23) found the NIR regression correlation coefficients for saturated FA such as palmitic and stearic acids in rapeseed were lower (<0.8) that those for unsaturated FA, which were between 0.84 and 0.98 (25). Kohler and Kallweit (21) found that the NIR regression correlation coefficients for the 15 major FA in sheep intramuscular fat ranged from r = 0.57 to 0.90 (R^2: 0.32–0.81). When FTIR was used to predict the FA composition of dissected but unextracted adipose tissue (26) it gave poor correlation coefficients for the main FA (R^2 = 0.69–0.79) although much better correlations (R^2 = 0.91–0.98) were found if the tissue was extracted before analysis. Similarly, NIR analysis had poorer prediction ability for the less significant FA compared with Raman analysis (21).

It is clear that Raman has the advantage of giving correlation coefficients as high as the best of the other spectroscopic methods, and it can do this without the need for prior solvent extraction steps in the analysis. The most common method of determining the FA profile of adipose tissue and similar TG systems is to use GC; typically, 100-m capillary columns and temperature ramps are required to obtain effective separation and identification of isomers. Since this GC method requires time-consuming, destructive sample extraction and transmethylation, the ability to use Raman spectroscopy, with a simple calibration, on unextracted, dissected tissue is a significant advantage.

Thus, Raman spectroscopy can accurately predict the bulk properties of adipose tissue and a detailed FA profile of the 18 most abundant FA found in neat adipose tissue with an accuracy at least as good as that of other spectroscopic methods but with extremely straightforward sampling. The bulk unsaturation parameters could be predicted successfully (*cis*: RMSEP = 4.7% of 4 σ; total PUFA RMSEP = 4.0% of 4 σ) but *trans* unsaturation was not so well modeled (RMSEP = 18% of 4 σ). For the individual FA, the average RMSEP of the 18 most abundant FA was 11.9% of 4 σ while the prediction errors for the five most abundant FA were all better than the average value (in some cases as low as RMSEP = 4.7% of 4 σ). Of particular note was the ability of Raman spectroscopy to predict quantities important in human health, such as the proportion of 18:3*c*Δ9,12,15 present, or the total proportion of PUFA. The accuracy of the prediction, although as good as other spectroscopic methods, does not approach that of conventional GC methods: This method is not intended as an alternative to established chromatographic methods for high-accuracy profiling. The approach is predominantly aimed at on-line applications, in which the lower accuracy is compensated by the ability to make rapid, noncontact measurements with no additional sample preparation.

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Fluorescent Image Analysis of Lipid Hydroperoxides in Fish Muscle with 3-Perylene Diphenylphosphine

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ABSTRACT: A fluorescent image analysis method was developed to evaluate lipid hydroperoxide formation in fish muscle. The lipid hydroperoxides generated in white and dark fish muscles during storage at 5–6°C oxidized 3-perylene diphenylphosphine located in the tissue to yield the fluorescent derivative, 3-perylene diphenylphosphine oxide (3-PeDPPO). 3-PeDPPO thus obtained was determined by digital fluorescent image analysis. The 3-PeDPPO fluorescence intensity of white and dark muscle increased during low-temperature storage (0–24 h) and was clearly correlated with total lipid hydroperoxide levels in muscle extracts, which were determined by using HPLC based on a triphenylphosphine oxidation method (R^2 = 0.954). These results suggest that 3-PeDPPO fluorescence, coupled with fluorescent image analysis, is a novel tool for direct determination of lipid hydroperoxides in fish muscle without a need for extraction of lipid.

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Lipid peroxidation is a major cause of the deterioration of fatty fish during storage because of its high content of PUFA. Several methods have been proposed to quantify hydroperoxides in food and biological systems, including an iodometric assay (1), complexation of Fe(III) (2), enzymatic reaction (3), and chromatographic techniques (4–6). Some significant investigations of lipid peroxidation have been carried out by reducing hydroperoxides with a triarylphosphine possessing a fluorophore instead of the phenyl group of triphenylphosphine (TPP), such as diphenyl-1-pyrenylphosphine (DPPP), and quantifying the resultant formation of the arylphosphine oxide by its intense fluorescence (7). However, DPPP has fluorescence excitation and emission wavelengths in the UV region, possibly causing lipid peroxidation when irradiated by itself. Recently developed advanced image analysis methods support visual subjective tests in the routine evaluation of food quality. Fluorescent image analysis is a selective and sensitive method that offers several advantages, including easy and accurate identification of structures, quantification at low concentrations, high analysis speed, and rapid sample preparation and determination (8). Although direct image analysis on cultured cells with DPPP oxide fluorescence has been reported (9–11), fluorescent compounds such as tocopherols will also interfere with the DPPP image analysis of meats because of overlapping fluorescence emissions. In this study, a novel fluorescent probe, 3-perylene diphenylphosphine (3-PeDPP) (12,13), was applied to a fluorescent image analysis for the quantification of lipid hydroperoxide (LOOH) generated in fish muscle during low-temperature storage.

In our previous reports, fluorescence intensity increased dramatically when 3-PeDPP was oxidized to 3-perylene diphenylphosphine oxide (3-PeDPPO), the corresponding oxidation product of 3-PeDPP, which has an excitation wavelength at 440 nm (12,13). In the present study, the 3-PeDPP fluorescent probe showed better selectivity in combination with fluorescent image analysis for quantification of LOOH. Successful measurement allows the possible application of 3-PeDPP with fluorescent image analysis as a novel tool to detect LOOH formation in fish muscle directly and satisfactorily.

MATERIALS AND METHODS

Chemicals. 3-PeDPP was prepared from 3-bromoperylene and TPP and identified as previously described (12,13), based on the method of Akasaka *et al.* (7). The purified 3-PeDPP was kept as the solid under nitrogen gas (N_2) in the dark at −20°C until use. The solution of 3-PeDPP was freshly prepared before the experiment to prevent 3-PeDPP oxidation. 2-Thiobarbituric acid and 2,6-di-*tert*-butyl-*p*-cresol (BHT) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Cumene hydroperoxide (CumOOH) and TPP were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Dimethyl sulfoxide was purchased from Wako Pure Chemical Industries (Osaka, Japan). PBS powder was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Other organic solvents and chemical reagents were of analytical grade and used without further purification. Commercial tuna oil (T.C. Union Food Co., Ltd., Bangkok, Thailand), soybean oil (Miyazawa Yakuhin Co., Ltd., Tokyo, Japan), and cashew nut oil (extracted in the laboratory) were used as sample oils. These oils were purified by open column chromatography on Spherical silica gel 60 40–50 μm (Kanto Chemical Co. Inc., Tokyo, Japan) by using *n*-hexane followed by 7% of diethyl ether in *n*-hexane.

Preparation of white and dark muscles. A sample of yellowtail (*Seriola quinqueradiata*) was purchased from a local fish market. The specimen was separated into white muscle and

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Abbreviations: a.u., arbitrary units; CumOOH, cumene hydroperoxide; DPPP, diphenyl-1-pyrenylphosphine; LOOH, lipid hydroperoxide; 3-PeDPP, 3-perylene diphenylphosphine; 3-PeDPPO, 3-perylene diphenylphosphine oxide; TPP, triphenylphosphine; TPPO, triphenylphosphine oxide.

dark muscle, taken separately from the fillet under the lateral line, and these were immediately cut into two pieces, about 10 g each, in sizes of 4.0 × 2.0 × 0.6 cm. Pieces of white and dark muscles were placed in an empty petri dish (60 × 15 mm polystyrene; Becton Dickinson Labware, Franklin Lakes, NJ), one piece per dish, and stored in the dark at 5–6°C.

Determination of total lipid content. Total lipid content in white and dark muscle was determined by the method of Bligh and Dyer (14).

Determination of PV by iodometric method. PV of oils was determined by an iodometric method according to AOCS Official Method Cd 8-53 (15).

Determination of LOOH with 3-PeDPP. Oil samples were prepared by dissolving 5–10 mg of sample in 25 mL of a mixture of chloroform and methanol (1:1, vol/vol). Then 2.9 mL of the oil solution was mixed with 0.1 mL 3-PeDPP in methanol at a final concentration of 75 μM in a glass reaction vessel (Reacti-vial; Pierce, Rockford, IL). The vial was tightly capped and kept at room temperature for 30 min in the dark. The fluorescence due to 3-PeDPPO was measured at excitation and emission wavelengths of 440 and 470 nm, respectively, with a model RF-1500 spectrofluorometer (Shimadzu, Kyoto, Japan).

Determination of LOOH based on TPP oxidation. On the basis of TPP oxidation, LOOH levels were determined by HPLC as described by Nakamura and Maeda (16). Lipids were extracted from stored samples by chloroform/methanol (2:1, vol/vol) containing 0.05% (wt/vol) BHT, which was added to prevent lipid peroxidation during extraction. Lipid samples (50 mg) were dissolved in 10 mL of cyclohexane in a glass reaction vessel and reacted with 800 μM TPP at 30°C for 30 min in the dark. Triphenylphosphine oxide (TPPO), a reaction product of TPP and LOOH, was separated by HPLC on a Mightysil RP-18GP column (4.6 × 250 mm, 3 μm film thickness; Kanto Chemical Co. Inc., Tokyo, Japan) using a mixture of CH_3CN and water (9:1 vol/vol) as a mobile phase at a flow rate of 1.0 mL per min and detected at 260 nm with an SPD 10Av UV-vis detector (Shimadzu). The content of LOOH was calculated from the peak area of TPPO and was expressed as nanomoles LOOH per gram of muscle. A calibration curve was used to convert peak area into concentration of CumOOH equivalent (μM). Finally, levels of LOOH were expressed as nanomoles of CumOOH equiv/gram of fish muscle (nmol CumOOH equiv/g muscle).

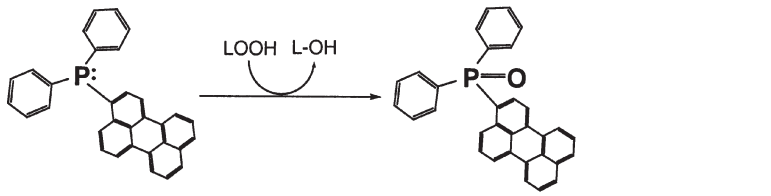
Determination of TBARS. Accurately weighed fish muscle (2.5 g) was homogenized in 10.0 mL of PBS (275–285 mOsm/kg, pH 7.4) using an Ace homogenizer model AM-3 (Nihonseiki Kaisha, Tokyo, Japan). The homogenate was used for TBARS assay. The TBARS were determined by the method of Rice-Evan *et al.* (17). Absorption was measured at 532 nm with a UV-vis spectrophotometer (model UV-1700; Shimadzu) and using a molar extinction coefficient of 156×10^3 M^{-1} cm^{-1} for calculating TBARS concentration (17). TBARS were expressed as nanomoles of malondialdehyde per gram muscle.

Analysis of LOOH by fluorescent image analysis. For fluorescent image analysis, the white and dark muscle sections (about 0.5 mm thickness) were individually taken at appropriate intervals (0–24 h) and stained in a mixture of PBS (8,000 mg/L of NaCl, 200 mg/L of KCl, 1,150 mg/L of $NaHPO_4$ and 200 mg/L of KH_2PO_4, 275–285 mOsm/kg, pH 7.4) and DMSO (9:1, vol/vol) containing 3-PeDPP at a final concentration of 1.2 μM for 30 min at room temperature in the dark. The fluorescent images of LOOH generated in fish muscle were obtained with an Olympus IX70 inverted microscope (Olympus, Tokyo, Japan) equipped with an Olympus 40X UApo/340 (40-fold, N.A. = 0.90; Olympus, Tokyo, Japan) as an objective lens and an Olympus UMNBV cube filter unit (420–440 nm excitation, 475 nm emission). Image data were digitally acquired with an Olympus model C-3040 Camedia digital camera equipped with a NY-2000S eyepiece-mount adapter (MeCan Imaging Inc., Saitama, Japan) according to the manufacturer's instruction. The acquired data were converted into 8-bit gray-scale digital data by measuring mean gray scale pixel densities with ImageJ software (National Institutes of Health, Bethesda, MD). The resulting mean pixel densities were used as fluorescence intensities at arbitrary units (a.u.).

Statistical analysis. Student's *t*-test was used to distinguish significant differences among the mean values. A statistically significant difference was declared at $P < 0.05$.

RESULTS

Detection of LOOH with 3-PeDPP. The reaction for 3-PeDPPO formation is shown in Scheme 1. In the present study, under a temperature of 37°C and with incubation for 0–40 min, 3-PeDPP was also oxidized to 3-PeDPPO in various oils and oxidized oils (Fig. 1A). 3-PeDPP at a final concentration of 75 μM in the reaction solutions was reacted with tuna oil, soybean oil, and cashew nut oil containing a range of 2–13 nanoequivalents CumOOH per 3 mL cuvette in a solution of chloroform/methanol (1:1, vol/vol) with continuous stirring and darkness at room temperature. The fluorescence intensity increased



SCHEME 1

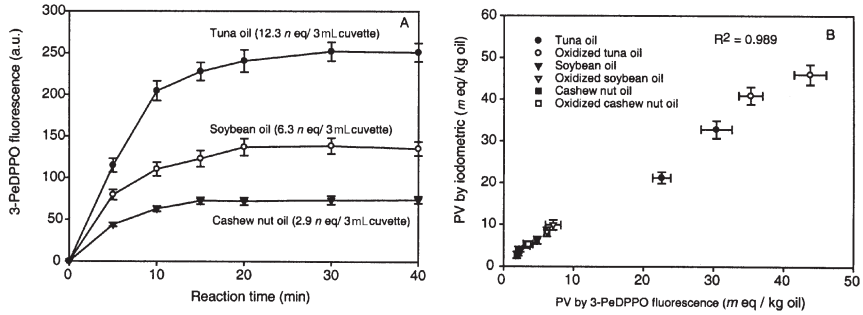


FIG. 1. (A) Time course of 3-perylene diphenylphosphine oxide (3-PeDPPO) formation with different concentrations of lipid hydroperoxide in various oil samples. The vertical bars represent the SD for data from 3-PeDPPO fluorescence (n = 3). (B) Relationship between PV obtained by the iodometric method and by the 3-PeDPPO-fluorescence method. The vertical bars represent the SD for data obtained by iodometric method (n = 3). The horizontal bars are the SD for data obtained by 3-PeDPPO fluorescence method (n = 3). Abbreviation: a.u., arbitrary units.

exponentially for 10 min due to the formation of 3-PeDPPO, which was stable within 30 min (Fig. 1A).

The PV of various oils and oxidized oils were determined by using the 3-PeDPPO fluorescence and the iodometric method. The oxidized oils were prepared by incubating the oils in an air oven set at 40°C for 1 h. Figure 1B shows a good linear regression between the results obtained by a traditional iodometric method and the proposed novel 3-PeDPP assay (R^2 = 0.989).

Application of 3-PeDPPO fluorescence image analyses of fish muscle lipid oxidation. The results for the total lipid content from white and dark muscles of yellowtail revealed no significant differences (8.2 and 8.5% for white and dark muscle, respectively). The contents of total LOOH and TBARS in white and dark muscle of yellowtail stored at 5–6°C for 0–24 h were determined (Fig. 2). Dark muscle contained a significantly higher amount of total LOOH and TBARS compared with white muscle ($P < 0.05$) from 6 to 24 h under storage at 5–6°C. The total LOOH contents in the muscle increased from 24.3 ± 2.8 to 174.0 ± 23.7 and from 53.5 ± 10.2 to 485.0 ± 30.7 nmol per g of white and dark muscle, respectively. The TBARS contents in the muscle also increased from 1.2 ± 0.08 to 3.8 ± 0.91 and from 2.2 ± 0.07 to 11.5 ± 2.0 nmol per g of white and dark muscle, respectively.

The digital images of 3-PeDPPO fluorescence in white and dark muscle slices stored at 5–6°C for 0–24 h are shown in Figure 3. Strong fluorescence due to 3-PeDPPO was observed in white and dark muscle after 12 and 6 h, respectively. 3-PeDPPO fluorescence determined through digital image analysis is shown in Figure 4. Strong fluorescence (7.9 ± 2.7 to 130.4 ± 17.2 a.u.) due to 3-PeDPPO was observed in the dark muscle, whereas weak fluorescence (3.6 ± 1.1 to 61.5 ± 17.2 a.u.) was obtained in the white muscle. The fluorescent analysis data also showed a good correlation with total LOOH content obtained by the TPP oxidation method (R^2 = 0.954) (Fig. 5).

DISCUSSION

The reaction of triarylphosphine with hydroperoxide or a hydroperoxy group seems to be a general phenomenon of triarylphosphines having a fluorophore instead of the phenyl group of TPP or fluorescent probes based on fluorophores in TPP analogs (18). Fluorescence detection based on a fluorophore in TPP analogs with a HPLC method of post-column

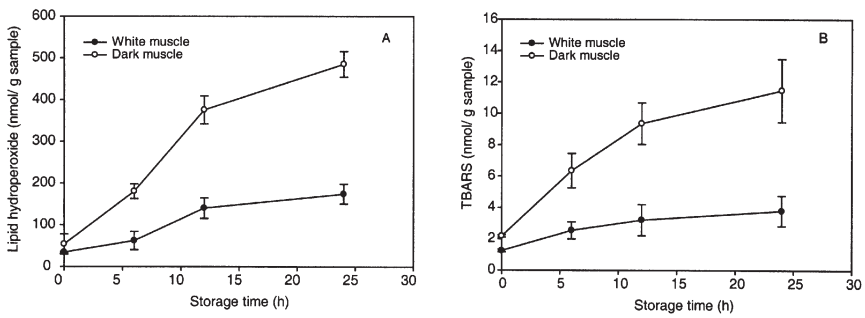


FIG. 2. Changes in lipid hydroperoxides (A) and TBARS (B) formation in white and dark muscle during storage at 5–6°C. The vertical bars represent the SD (n = 3).

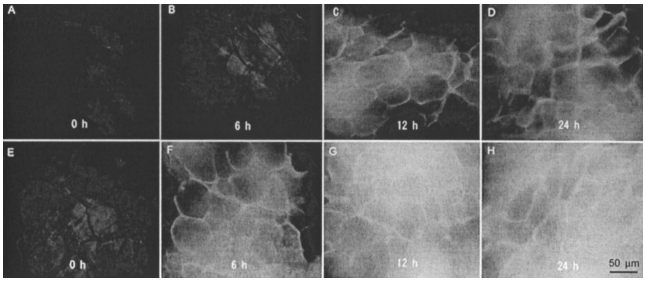


FIG. 3 . Typical 3-PeDPPO fluorescence images from 1.2 µM 3-perylene diphenylphosphine-labeled fish muscle slices. The visible light lines in the panels delineate cell membranes of white (panels A–D) and dark (panels E–H) muscle during storage at 5–6°C, respectively. For abbreviation see Figure 1.

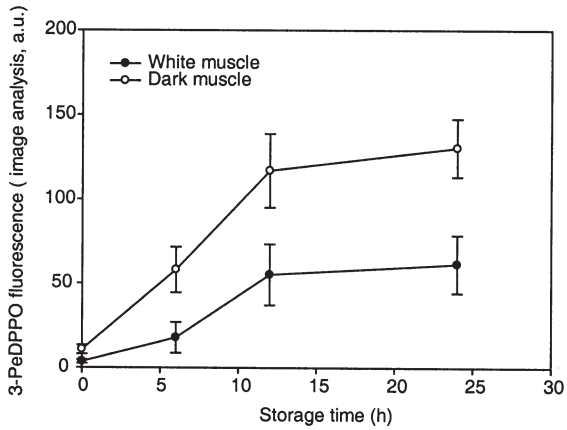


FIG. 4. 3-PeDPPO fluorescence intensity (a.u.) from fluorescent image analysis of white and dark muscle (Fig. 3) during storage at 5–6°C. The vertical bars are the SD of 3-PeDPPO fluorescence in each image field. For abbreviations see Figure 1.

derivatization was developed for improving the specificity and sensitivity over the other available methods (5,18,19). However, these methods need uniformity of oil samples; and complicated sample preparation procedures, such as homogenization and lipid extraction of oil-containing materials, are necessary before determination of LOOH. Moreover, LOOH are difficult to measure accurately because of their short lifetime.

Direct fluorescent image analysis is a selective and sensitive method that offers several advantages such as easy and accurate identification of structures and rapid preparation and determination (8). Direct fluorescent image analysis without sample preparation procedures was carried out with a fluorescent probe based on the fluorophore in TPP analogs such as DPPP oxide fluorescence (9–11) and had several advantages such as the possibility of continuous observation of oxidation in cells in a nondestructive manner. However, the disadvantage of DPPP oxide image analysis may be that DPPP oxide has a fluorescence emission wavelength of 380 nm in the UV region, causing lipid peroxidation and cell death by itself. Moreover, the presence of tocopherol analogs will interfere with DPPP oxide image analysis of lipid-containing fluorescent compounds because of overlapping fluorescence.

3-PeDPP is a nonfluorescent compound, but when it is oxidized to 3-PeDPPO, the resultant oxidation products exhibit dramatically increased fluorescence intensities, with excitation (440 nm) and emission (470 nm) wavelengths (12,13). This increase in fluorescence intensity is due to decreases in steric hindrance around the phosphorus atom and increases in electron density on the phosphorus atom (20).

The fluorescence intensity due to 3-PeDPPO increased proportionally with increasing concentration of hydroperoxides generated in homogeneous solutions, as well as in nonhomogeneous solutions, such as phospholipid liposome models oxidized with the free radical generator 2,2′-azobis(2-amidinopropane)dihydrochloride and photosensitized oxidation (12). 3-PeDPP as the fluorescent probe coupled with fluorescent image analysis enabled us to observe the fluorescence due to the reaction between 3-PeDPP and LOOH in cell membranes and thin slices of liver and heart tissues in a nondestructive manner (12).

As shown in Figure 1A, the fluorescence intensity of 3-PeDPPO increased dramatically with higher reaction times and amounts of LOOH from various types of oil. For the 3-PeDPPO fluorescence method, 5–10 mg of lipid was sufficient for a single assay, but the iodometric method required the use of oil on a gram scale for the determination. In our previous report (13), the kinetic profiles of LOOH formation were investigated by both 3-PeDPPO fluorescence and ferric-xylenol orange methods during autoxidation of refined tuna oil at 40°C. In the present study a linear correlation (coefficient of determination R^2 = 0.989) was found between lipid hydroperoxide contents in various oils and oxidized oils as determined by 3-PeDPPO fluorescence and by the iodometric method. (Fig. 1B). This result suggests that 3-PeDPPO fluorescence could be used as a sensitive tool for quantitative determination of trace

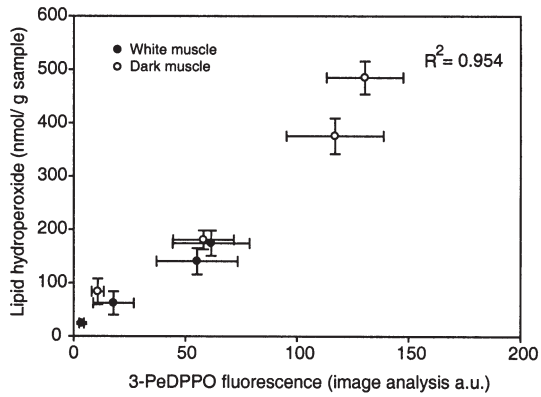


FIG. 5. Relationship between lipid hydroperoxide levels in white and dark muscle during low-temperature storage determined by HPLC based on oxidation of triphenylphosphine and by 3-PeDPPO fluorescence (a.u.) from image analyses. Data are represented as mean ± SD (n = 3). The horizontal bars are the SD for data obtained by the 3-PeDPPO fluorescence method, and the vertical bars represent the SD for the triphenylphosphine method. For abbreviations see Figure 1.

amounts of LOOH in various oils, which supports the performance of the 3-PeDPP assays.

It is well established that lipid peroxidation is an important contributor to postmortem deterioration of fish quality during storage. Fish meat is more susceptible to oxidative degradation than other meats during storage because of the abundance of PUFA in the tissues. LOOH are the primarily oxidative products of unsaturated FA (21). Changes in the content of LOOH generated in white and dark muscles of yellowtail during low-temperature storage were successfully measured by the proposed 3-PeDPPO fluorescence image analyses, which were linearly correlated with the data by HPLC methods based on TPP oxidation (16).

The present results showed the enhanced production of LOOH and secondary lipid peroxidation product (TBARS) in dark muscle of yellowtail during low-temperature storage (0–24 h at 5–6°C) in comparison with those in white muscle (Fig. 2A and 2B, respectively). The results were in agreement with previous reports that the levels of the lipid peroxidation products in dark muscle were higher than those in the white muscle in wild and cultured yellowtail (22,23) and herring (24). This observation is due to the high contents of myoglobin in muscle intracellular structure or red muscle fibers (25). During the handling and storage of fish, a number of biochemical changes occur, leading to the formation of metmyoglobin (26,27). Metmyoglobin formation is positively correlated with lipid peroxidation (28,29), and high concentrations of myoglobin and other heme compounds in dark meats function as prooxidants in the muscle tissue (30).

Digital image analysis techniques are generally used to uncover information from image data. The information is derived by transforming the digital image or by directly extracting data values from the image. We carried out fluorescent image analysis for evaluation of LOOH formation in thin sections of yellowtail white and dark muscle during storage at 5–6°C. The visible light lines in Figure 3 delineate the cell membranes of white and dark muscle cross-sections during low-temperature storage. Strong fluorescence of 3-PeDPPO was observed in cell membranes of dark muscle after 6 h of low-temperature storage, but low fluorescence intensity of 3-PeDPPO was obtained in cell membranes of white muscle (Fig. 3). Moreover, the image analysis data also showed the good correlation with the data for LOOH by HPLC based on the TPP oxidation method (R^2 = 0.954, Fig. 5). Thus, 3-PeDPPO fluorescence in combination with fluorescent image analysis was successfully used to estimate 2-D LOOH formation in fish muscle during storage.

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Acyl Migration During Debenzylation of 1,3-Di-*O*-benzyl-2-*O*-acylglycerols

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ABSTRACT: In investigating the preparation of 2-acylglycerol by debenzylation of 1,3-di-*O*-benzyl-2-*O*-acylglycerol, twelve 1,3-di-*O*-benzyl-2-*O*-acylglycerols were synthesized. 2-*O*-Acyl moieties with one or more protons at the carbon atom adjacent to carbonyl group were found to migrate from C-2 to C-1 of the glycerol moiety during hydrogenation under Pd/C at room temperature.

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A number of methods are available to prepare 2-*O*-protected glycerols. The method based on 1,3-*O*-benzylideneglycerol (1,2) results in low yields (3) owing to competitive formation of 1,2-*O*-benzylideneglycerol isomer, and its use would be expected to be restricted to 2-protecting groups stable to acid. The method based on 1,3-di-*O*-tritylglycerol is also unsuitable for acid-labile 2-protecting groups (4). The method based on 1,3-di-*O*-benzylglycerol (Fig. 1) appears to be compatible with different protecting groups. This method is the mildest and cheapest strategy to prepare 2-*O*-acylglycerols and has been used to prepare 2-*O*-acetyl, benzoyl, and *p*-toluenesulfonyl glycerol (5,6).

In our research on preparing 2-*O*-acylglycerols *via* debenzylation of 1,3-di-*O*-benzyl-2-*O*-acylglycerols, we found that some acyl groups migrated to a limited extent (acetyl) or entirely (stearyl) (Fig. 1). The intramolecular migration of acyl groups in MAG and DAG has been studied (7,8). Bases or acids catalyze this reaction (9,10). There is also a report on the solid-state isomerization of partial acylgycerols (11). The acyl migration under various conditions of a palmitoyl group in 1,2-dipalmitoyl-*sn*-glycerol to form 1,3-dipalmitoylglycerol was studied (12). However, the acyl migration during hydrogenation under Pd/C and the role of acyl characters on this migration were not reported. To investigate the parameters affecting the preparation of 2-acylglycerol from 1,3-di-*O*-benzyl-2-*O*-acylglycerol, twelve 1,3-di-*O*-benzyl-2-*O*-acylglycerols were synthesized and the acyl migration during debenzylation was studied.

EXPERIMENTAL PROCEDURES

Instrumentation. ^{1}H NMR spectra were obtained on a Bruker Avance 600 NMR spectrometer (Bruker, Rheinstetten, Germany) and Varian Unity Inova 400 WB Superconducting NMR Spectrometer (Varian Inc., Palo Alto, CA). Chemical shifts (δ) are given in ppm relative to internal standard tetramethylsilane and coupling constants in Hertz (Hz).

Materals and reagents. Silica gel for column chromatography (Silica gel H 200–300 mesh) and for TLC plates (Silica gel GF_{254} 10–40 μm) was purchased from Qingdao Haiyang Chemical Ltd., Co. (Qingdao, China). All products were purified by silica gel column chromatography before NMR analysis.

*1,3-Di-O-benzylglycerol (**1**)*. A solution of sodium hydroxide (30 g, 0.75 mol) in water (100 mL) was added over 10 min to benzyl alcohol (110 g, 1.06 mol). The mixture was cooled to 25°C, and then epichlorohydrin (30.6 g, 0.331 mol) was added under vigorous stirring over 30 min. Stirring was continued for 16 h. The mixture was then diluted with water (200 mL) and extracted with EtOAc (3 × 400 mL). The EtOAc layer was washed with water (500 mL), dried over anhydrous Na_2SO_4, and evaporated to give a liquid, which was distilled under reduced pressure to afford 56.3 g of **1** with a yield of 63%.

*General procedure for preparation of 1,3-di-O-benzyl-2-O-acylglycerol (**2a–2l**)*. A solution of 1 equiv acyl chloride in $CHCl_3$ was added to a mixed solution of 1 equiv 1,3-*O*-benzylglycerol and 1.1 equiv Et_3N in $CHCl_3$. The mixture was stirred for 2 h at room temperature and then washed with 1 M HCl (aq) and brine, dried over anhydrous Na_2SO_4, and evaporated to give **2a–2l** as a pale yellow liquid (Table 1).

*General procedure for preparation of 1-O-acylglycerol or 2-O-acylglycerol (**3a–3l**)*. A mixture of **2** and 5% Pd/C (10 mol%) in ethanol was stirred at room temperature under H_2 for 12 h, and filtered. The filtrate was evaporated to give **3** as a pale yellow liquid with yield >98% (Table 2).

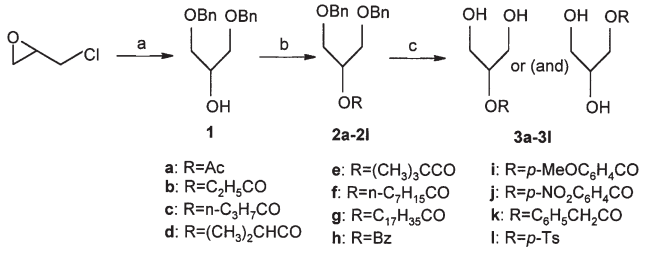

FIG. 1. Synthesis of 2-acylglycerol from 1,3-di-*O*-benzyl-2-*O*-acyl glycerol. Reaction a: Benzyl alcohol (BnOH), NaOH, MeOH, reflux 8 h, 63% yield; reaction b: acyl chloride, Et_3N, $CHCl_3$, 76–96% yield; reaction c: H_2, 5% Pd/C, 12 h, >98% yield. *p*-Ts = *p*-toluenesulfonyl.

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TABLE 1
Preparation of 1,3-Di-*O*-benzyl-2-*O*-acylglycerol

Compd.	Yield (%)	^{1}H NMR data in $CDCl_3$ at 600 MHz, δ (multiplicity, *J* = Hz)[a]
2a	96	2.07 (3H, *s*), 3.64 (4H, *d*, 5.2), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.20 (1H, *quint*, 5.2), 7.30 (10H, *m*)
2b	97	1.15 (3H, *t*, 7.6), 2.35 (2H, *q*, 7.6), 3.64 (4H, *d*, 5.2), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.23 (1H, *quint*, 5.2), 7.26–7.33 (10H, *m*)
2c	95	1.15 (3H, *t*, 7.6), 1.64 (2H, *sext*, 7.6), 2.31 (2H, *t*, 7.6), 3.64 (4H, *d*, 5.2), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.23 (1H, *quint*, 5.2), 7.25–7.32 (10H, *m*)
2d	92	1.17 (6H, *d*, 6.9), 2.58 (1H, *sept*, 6.9), 3.64 (4H, *d*, 5.2), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.23 (1H, *quint*, 5.2), 7.24–7.35 (10H, *m*)
2e	76	1.17 (9H, *s*), 3.64 (4H, *d*, 5.2), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.23 (1H, *quint*, 5.2), 7.23–7.32 (10H, *m*)
2f	97	0.85 (3H, *t*, 7.2), 1.28 (10H, *m*), 2.33 (2H, *t*, 7.6), 3.64 (4H, *d*, 5.2), 4.50 and 4.54 (each 2H, 12.2), 5.23 (1H, *quint*, 5.2), 7.24–7.33 (10H, *m*)
2g	96	0.88 (3H, *t*, 6.8), 1.28 (30H, *m*), 2.33 (2H, *t*, 7.5), 3.64 (4H, *d*, 5.2), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.22 (1H, *quint*, 5.2), 7.25–7.31 (10H, *m*)
2h	85	3.79 (4H, *d*, 4.8), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.47 (1H, *quint*, 4.8), 7.22–7.32 (11H, *m*), 7.45 (2H, *m*), 8.06 (2H, *m*)
2i	82	3.77 (4H, *d*, 4.8), 3.86 (3H, *s*), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.43 (1H, *quint*, 4.8), 6.92 (2H, *d*, 8.7), 7.21–7.31 (10H, *m*), 8.01 (2H, *d*, 8.7)
2j	87	3.78 (4H, *d*, 4.8), 4.50 and 4.54 (each 2H, *d*, 12.2), 5.23 (1H, *quint*, 4.8), 7.24–7.35 (10H, *m*), 8.20 (2H, *d*, 8.8), 8.27 (2H, *d*, 8.8)
2k	93	3.74 (6H, *m*), 4.57 (4H, *m*), 5.36 (1H, *m*), 7.22–7.37 (15H, *m*)
2l	94	2.36 (3H, *s*), 3.64 (4H, *d*, 7.2), 4.40 and 4.44 (each 2H, *d*, 12.2), 4.75 (1H, *quint*, 7.2), 7.21 (6H, *m*), 7.30 (6H, *m*), 7.76 (2H, *d*, 6.4)

[a]^{1}H NMR spectra of **2a**, **2g**, **2h**, and **2l** were recorded at 400 MHz.

RESULTS AND DISCUSSION

The substituents on the phenyl ring of the acyl groups (**2i** and **2j**) as well as steric hindrance (**2d** and **2e**) have no effect on acyl migration in 1,3-di-*O*-benzyl-2-*O*-acylglycerol under hydrogenation. The length of the side chain in an aliphatic acyl group has little effect on acyl migration (**3a–3d**, **3f**, **3g**). Thus, 2-*O*-acyl groups with one or more protons at the C-2 position in the acyl moiety are inclined to migrate during debenzylation. It could be estimated that H-2 in the acyl moiety may be encountered in the acyl migration. The proposed mechanism, as depicted in Figure 2, is different from the migration of 2-*O*-acyl to

TABLE 2
2-*O*-Acylglycerol (A) and/or 1-*O*-Acylglycerol (B) Prepared by Hydrogenation of 1,3-Di-*O*-benzyl-2-*O*-acylglycerol

Compd.	A/B[a] (mol/mol)	^{1}H NMR data in $CDCl_3$ at 600 MHz, δ (multiplicity, *J* = Hz)[b]
3a	1:2	A: 2.12 (3H, *s*), 3.82 (4H, *d*, 6.0), 4.90 (1H, *quint*, 6.0) B: 2.10 (3H, *s*), 3.60 and 3.70 (each 1H, *dd*, 13.0, 6.0), 3.93 (1H, *m*), 4.13 (1H, *dd*, 12.0, 9.0), 4.18 (1H, *dd*, 12.0, 10.8)
3b	0:100	1.15 (3H, *t*, 7.6), 2.32 (2H, *q*, 7.6), 3.60 (1H, *dd*, 12.0, 6.0), 3.72 (1H, *dd*, 12.0, 4.0), 3.97 (1H, *quint*, 4.8), 4.16 (1H, *dd*, 12.0, 7.3), 4.21 (1H, *dd*, 12.0, 4.7)
3c	0:100	1.15 (3H, *t*, 7.6), 1.64 (2H, *sext*, 7.6), 2.31 (2H, *t*, 7.6), 3.60 (1H, *dd*, 12.0, 7.3), 3.70 (1H, *dd*, 12.0, 5.8), 3.94 (1H, *m*), 4.16 (1H, *dd*, 13.3, 7.3), 4.21 (1H, *dd*, 11.6, 4.7)
3d	0:100	1.19 (6H, *d*, 6.9), 2.61 (1H, *sept*, 6.9), 3.60 (1H, *dd*, 11.6, 4.0), 3.70 (1H, *dd*, 11.6, 5.8), 3.94 (1H, *quint*, 6.0), 4.16 (1H, *dd*, 12.0, 5.0), 4.21 (1H, *dd*, 12.0, 6.0)
3e	100:0	1.15 (9H, *s*), 3.82 (4H, *d*, 4.8), 5.11 (1H, *quint*, 4.8)
3f	0:100	0.98 (3H, *t*, 7.2), 1.38 (10H, *m*), 2.33 (2H, *t*, 7.5), 3.60 (1H, *dd*, 12.0, 6.0), 3.70 (1H, *dd*, 11.6, 4.0), 3.94 (1H, *quint*, 6.0), 4.16 (1H, *dd*, 12.0, 6.0), 4.21 (1H, *dd*, 11.6, 4.7)
3g	0:100	0.88 (3H, *t*, 7.2), 1.28 (12H, *m*), 2.33 (2H, *t*, 7.5), 3.60 (1H, *dd*, 13.3, 7.3), 3.70 (1H, *dd*, 11.6, 5.8), 3.94 (1H, *m*), 4.16 (1H, *dd*, 13.3, 7.3), 4.21 (1H, *dd*, 11.6, 4.7)
3h	100:0	3.97 (4H, *m*), 5.17 (1H, *m*), 7.45 (2H, *t*, 8.0), 7.58 (1H, *t*, 7.6), 8.05 (2H, *d*, 8.0)
3i	100:0	3.85 (3H, *s*), 3.90 (4H, *d*, 4.6), 5.11 (1H, *quint*, 4.6), 6.90 (2H, *d*, 8.6), 8.00 (2H, *d*, 8.6)
3j	100:0	3.90 (4H, *d*, 4.9), 4.59 (1H, *quint*, 4.9), 6.51 (2H, *d*, 8.5), 7.86 (2H, *d*, 8.5)
3k	9:20	A: 4.86 (1H, *quint*, 4.8), 3.70 (4H, *m*), 3.63 (2H, s), 7.20–7.30 (5H, *m*) B: 3.57 (1H, *dd*, 11.0, 4.0), 3.45 (1H, *dd*, 11.0, 6.0), 4.09 (2H, *d*, 6.0), 3.61 (2H, *s*), 7.20–7.30 (5H, *m*)
3l	100:0	2.46 (3H, *s*), 3.80 (4H, *m*), 4.59 (1H, *m*), 7.36 (2H, *d*, 8.4), 7.84 (2H, *d*, 8.4)

[a]The ratio was determined by integration of the ^{1}H NMR signals for H-2 in the glycerol moiety.
[b]^{1}H NMR spectra of **3g**, **3h**, and **3l** were recorded at 400 MHz.

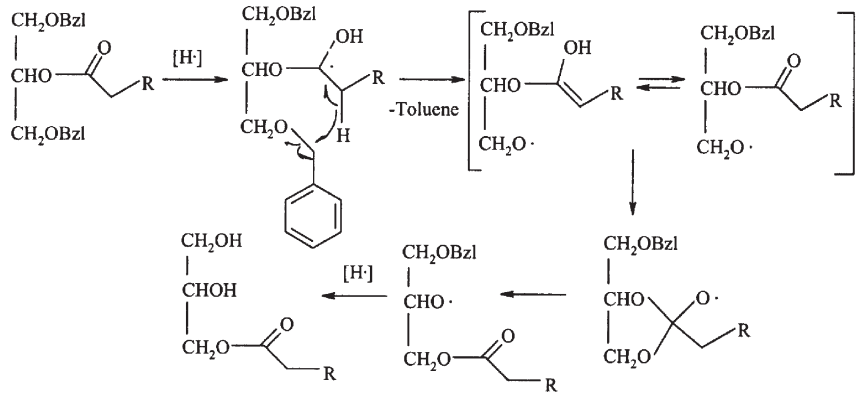

FIG. 2. Proposed mechanism for 2-acyl migration in 1,3-di-*O*-benzyl-2-*O*-acylglycerol under hydrogenation.

C-1 in acylglycerols *via* an ortho ester (11) or stabilized acyloxonium ion mechanism (13). Consequently, debenzylation of 1,3-di-*O*-benzyl-2-*O*-acylglycerol is only suitable for the preparation of 2-*O*-acylglycerols in which acyl groups possess no protons at the C-atom adjacent to carbonyl groups.

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Very Long Chain Fatty Acid Methyl Esters in Transesterified Palm Oil

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ABSTRACT: Some unidentified minor compounds have been observed in the residue from short-path distillation of transesterified palm oil that are not detected in the original palm oil. A method combining short-path distillation to enrich the unknowns with fractionation using solid-phase extraction is described. The fractionated components were identified using GC coupled with MS. The transesterified palm oil was found to contain methyl esters of up to C_{32} carbon atoms. In the very long chain FAME with carbon numbers ≥20, both even and odd carbon numbers accounted for 0.26 wt%, with C_{24} and C_{26} being the major ones present in the residue after short-path distillation of transesterified palm oil.

Paper no. L9884 in *Lipids 41*, 305–308 (March 2006).

Very long chain FA (VLCFA) with more than 20 carbon atoms are present in most common seed oils, albeit in small amounts (1). Sunflower oil contains three main saturated VLCFA: arachidic (20:0), behenic (22:0), and smaller amounts of lignoceric (24:0) acids. These FA account for 1–3% of the total FA in sunflower seeds (2). Recent analytical instrumentation has proved the occurrence of straight-chain odd-numbered FA as minor constituents in practically every natural fat (3). A detailed review of these VLCFA has been reported (4). Christie (5) also reported that all the possible odd-numbered homologs with 2–36 carbon atoms have been found in nature in esterified form.

Transesterification is a direct conversion of FA-containing lipids to alkyl esters by alcohol in the presence of a catalyst. The alkyl esters (particularly methyl esters) are produced from vegetable oils such as rapeseed oil (6,7), soybean oil (8,9), sunflower oil (10), and palm oil (11,12).

The FAME in palm oil and its products are expected to closely resemble the corresponding FA composition of crude palm oil (CPO), with up to C_{20} (13). VLCFA with more than 20 carbon atoms have not been reported in palm oil (14). However, some unidentified compounds have been observed in transesterified palm oil that are not found in the original oil. The main problems in identifying the minor components in transesterified palm oil are caused by their relatively low concentrations. Thus, they are unable to be detected using the current method of GC alone. An effort was made in this study to enrich the minor components using short-path distillation by removal of the bulk methyl esters.

A further enrichment was carried out using solid-phase extraction (SPE) to separate the mixture into groups of components. This can eliminate the interfering components and thus concentrate other trace components. Identification of the components was carried out using GC coupled with MS (GC–MS). The identification of FAME using GC has been described as a reliable tool, as the FAME show highly predictable behavior in GC, as reviewed by Ackman (15). Thus, the identification of very long chain FAME (VLCFAME) using GC–MS as the spectra provides complete information and makes possible the full identification of the methyl esters. If GC retention data are added, it is possible to be 90% certain of the identity of the FA. Some limited information on the positions of structural features, such as the double bond in polyenic FA, branch points, and oxygenated groups, also may be obtained (16). For separation of a mixture of methyl esters with a high molar mass, GC can be used up to C_{40} (4). Even- and odd-numbered methyl esters of up to 31:0 are available commercially; thus, we were able to carry out comparisons of retention times with standards.

EXPERIMENTAL PROCEDURES

Materials. CPO was obtained from the MPOB Palm Oil Mill Technology Centre in Labu, Negeri Sembilan, Malaysia.

Standard and chemical reagents. All solvents and chemicals used for transesterification, SPE, and analyses were of analytical or chromatographic grade, purchased from Merck (Darmstadt, Germany). A FAME mix (189201AMP) and saturated straight-chain FAME (ME7–1KT) were purchased from Supelco (Bellefonte, PA).

Method. The CPO was transesterified to palm oil methyl esters using the method described by Choo *et al.* (17). CPO (1 kg) was weighed into a (2-L) three-necked round-bottomed flask, and methanol (500 mL) was added to the flask. Sodium hydroxide (5.5 g) was dissolved in methanol and added to the flask. The mixture was refluxed at a temperature of 60–70°C for 1 h. The glycerol layer was separated and the methyl esters were washed with hot water until neutral. The methyl esters were dried with anhydrous sodium sulfate and subsequently pumped dry. The methyl esters were then subjected to short-path distillation (Pope Scientific Inc.) at a temperature of 150°C and pressure of <1.0 mTorr with a flow rate of 6.0–8.5 mL/min

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Abbreviations: CPO, crude palm oil; VLCFA, very long chain fatty acid(s); VLCFAME, very long chain FAME.

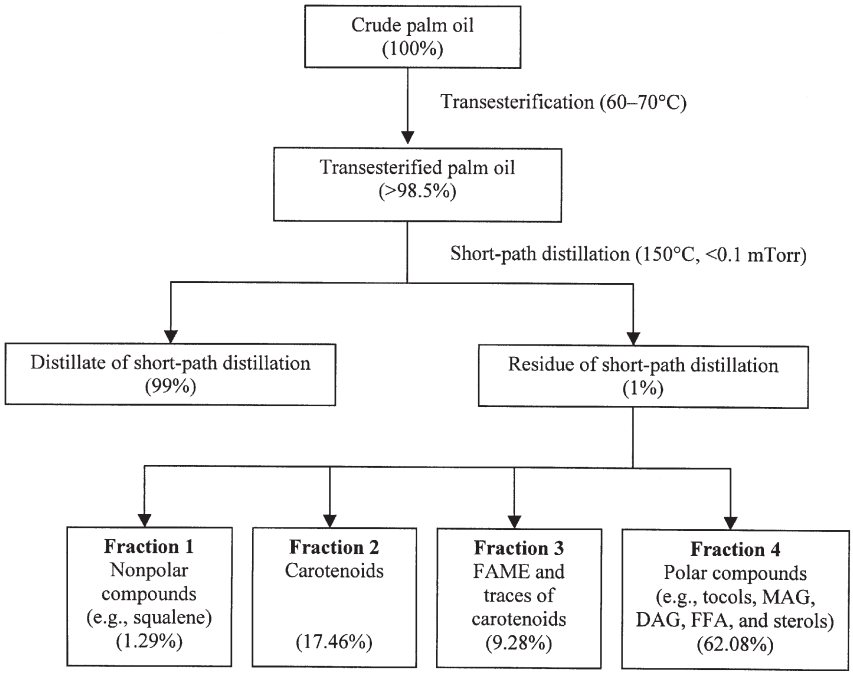

FIG. 1. Flow diagram of the enrichment and fractionation of methyl esters.

to obtain (i) distilled methyl esters and (ii) residue enriched with minor components. The residue was blanketed with nitrogen and stored in the dark at –20°C before use to prevent deterioration by heat, light, and air.

SPE. A Strata SI-1 1000 mg/6 mL (55 µm, 70 Å) silica gel column (Phenomenex, Torrance, CA) was rinsed with 2.0 mL of *n*-hexane. A 21.65-mg quantity of residue was weighed accurately and dissolved in 1.0 mL of *n*-hexane. The sample was transferred into the silica column and rinsed with 3.0 mL of *n*-hexane and eluted as fraction 1. The subsequent fractions 2 and 3 were eluted with 6.0 mL of *n*-hexane/toluene (85:15, vol/vol) each. Fraction 4 was eluted with 10.0 mL of methanol. The solvent was allowed to flow under gravity at a flow rate of 0.75 mL/min. The eluate was dried under nitrogen.

GC-FID. The sample was weighed accurately into a 2-mL GC vial. Then 1.0 mL of methylene chloride was pipetted into the vial. The instrument used was a Hewlett-Packard gas chromatograph, model 5890, series II (Avondale, PA), equipped with an FID and on-column injector. A fused-silica capillary column, BPX 5 (15 m × 0.32 mm) coated with (5% phenyl)-polysilphenylene-siloxane, film thickness of 0.25 µm (SGE, Austin, TX), was used. GC conditions were: injector temperature, 245°C; detector temperature, 370°C; initial oven temperature, 100°C; initial holding time, 1 min; ramping rate, 10°C/min; final temperature, 300°C; carrier gas (He) flow rate, 2 cm^3/min; column pressure, 14.5 psi; injection volume, 1 µL. Quantification was carried out using a five-point external standard calibration assay, with R^2 values > 0.99.

GC–MS. The instrument used was a Hewlett-Packard gas chromatograph, model 6890 series, coupled with a Hewlett-Packard mass selective detector, model 6890 series. The capillary column used was an HP-5MS coated with (5%-phenyl)-methylpolysiloxane (Agilent Technologies, Palo Alto, CA). The injector temperature was 245°C and the GC–MS interphase temperature was 280°C. The initial temperature of 100°C was held for 1 min and the final temperature of 280°C was held for 16 min. The ramping rate was 10°C/min. The mode of ionization was EI with an ionization voltage of 70 eV.

RESULTS AND DISCUSSION

The FAME in palm oil and its products are expected to closely resemble the corresponding FA composition of CPO. This paper reports the presence in transesterified palm oil of VLCFAME that include even and odd carbon number identified by (i) enriching the minor components by removing the bulk methyl esters using short-path distillation and (ii) consequent fractionation of the residue using SPE. Figure 1 shows a flow diagram of the enrichment and fractionation of the methyl esters. The yield in the figure shows the mean value of three replicates.

The presence of methyl esters in the residue obtained by short-path distillation of transesterified palm oil was confirmed with IR spectra. It showed the presence of a carbonyl functional group for esters, with C=O stretching at 1735 cm^{-1}. In addition, the C–H stretching for $–CH_3$ and $–CH_2–$ were shown at 2925 and 2855 cm^{-1}, respectively. These indicate the presence of saturated alkyl moieties in the residue.

The residue was then subjected to SPE for further isolation into groups of components. A total of four fractions were collected, with recovery ~90% (Fig. 1). When the fractions were analyzed, we found that the residue could be separated into four groups of components.

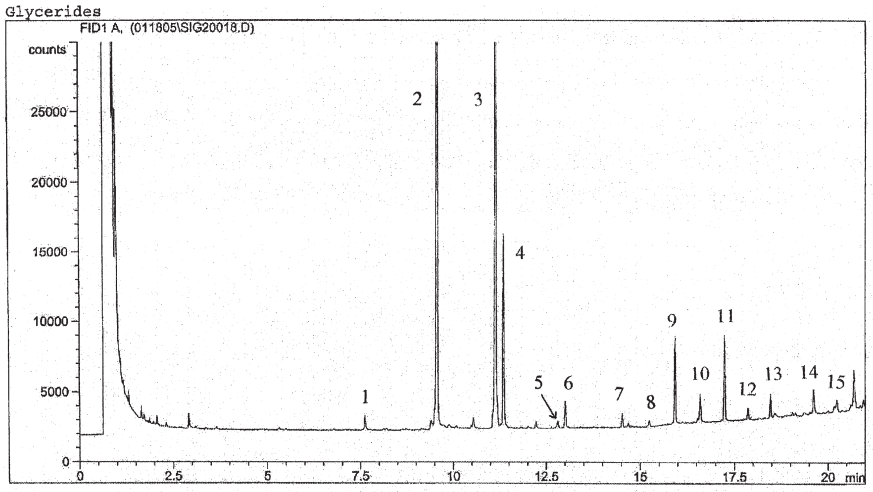

FIG. 2. Gas chromatogram of fraction 3.

The gas chromatogram of fraction 3 (Fig. 2) illustrates at least 15 identifiable peaks with good baseline separation. The mass spectra of the peaks show the characteristic peak in MS for saturated straight-chain methyl esters at *m/z* 74. This most abundant peak is the McLafferty rearrangement ion that involves a γ-hydrogen transfer followed by β-cleavage. The molecular ion at 382 is clearly seen for methyl esters of 24 carbon atoms. The ion at *m/z* 351, $[M - 31]^+$, arises following a simple homolytic cleavage of the methoxy bond to yield a commonly observed acylium ion. This ion confirms that the peak is indeed a methyl ester. Another abundant ion at *m/z* 87 shows a simple cleavage mechanism or fission of the bond between C3 and C4. An ion at *m/z* 339, $[M - 43]^+$, represents the loss of a C3 unit (carbons 2–4) *via* a complex rearrangement. Other prominent ions, including a $C_nH_{2n-1}O_2^+$ series (87, 101, 115, 129, 143, etc.) and the alkyl ion (57, 71, 85, etc.), also show the saturated straight-chain methyl ester.

The relative (%) concentration of the FAME identified in the residue of transesterified palm oil after short-path distillation is shown in Table 1. The quantification was carried out using a five-point external standard calibration assay, with R^2 values > 0.99. The residue from short-path distillation of transesterified palm oil was found to contain 2.50 wt% FAME ranging from C_{14} to C_{32} carbon atoms. The VLCFAME of >20 carbon atoms consisting of even and odd carbon numbers accounted for 0.26 wt%, with C_{24} and C_{26} being the major ones.

TABLE 1
Relative (%) Concentration of Methyl Esters Identified in Residue from the Short-Path Distillation of Transesterified Palm Oil

Peak no.	FAME	Relative concentration[a] (%)
1	14:0	0.54 ± 0.02
2	16:0	47.39 ± 0.05
3	18:1	34.02 ± 0.10
4	18:0	6.501 ± 0.005
5	20:1	0.27 ± 0.02
6	20:0	1.02 ± 0.03
7	22:0	0.54 ± 0.04
8	23:0	0.36 ± 0.01
9	24:0	2.89 ± 0.01
10	25:0	1.02 ± 0.04
11	26:0	3.06 ± 0.08
12	27:0	0.48 ± 0.05
13	28:0	0.933 ± 0.002
14	30:0	0.856 ± 0.008
15	32:0	0.12 ± 0.02

[a]Mean value of three replicates.

FAME of the residue from the short-path distillation of transesterified palm oil were satisfactorily separated from other interfering components for characterization and identification. However, because the ester moiety has similar properties, it was a challenge to isolate every individual constituent of the methyl esters. Nonetheless, GC–MS is considered a reliable and adequate tool for the identification of methyl esters (18).

The results of the present study clearly show that 2.50 wt% of FAME ranging from C_{14} to C_{32} carbon atoms, with both even and odd carbon atoms, were present in the residue from the short-path distillation of transesterified palm oil, whereas the VLCFAME with carbon atoms >20 accounted for 0.26 wt%.

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REVIEW

Natural Neo Acids and Neo Alkanes: Their Analogs and Derivatives

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ABSTRACT: This review presents more than 260 naturally occurring (as well as 47 synthesized) neo fatty (carboxylic) acids, neo alkanes, and their analogs and derivatives, isolated and identified from plants, algae, fungi, marine invertebrates, and microorganisms, that demonstrate different biological activities. These natural metabolites are good prospects for future chemical preparations as antioxidants, and also as anticancer, antimicrobial, and antibacterial agents. Described also are some synthetic bioactive compounds containing a tertiary butyl group(s) that have shown high anticancer, antifungal, and other activities. Applications of some neo fatty (carboxylic) acid derivatives in cosmetic, agronomic, and pharmaceutical industries also are considered. This is the first review to consider naturally occurring neo fatty (carboxylic) acids, neo alkanes, and other metabolites containing a tertiary butyl group(s) [or *tert*-butyl unit(s)].

Paper no. L9944 in *Lipids 41*, 309–340 (April 2006).

This review is a comprehensive survey of neo fatty (carboxylic) acids and/or their derivatives obtained from living organisms. FA constitute the most abundant class of natural compounds and are components of complex lipids. FA differ in their number of olefinic bonds, the extent of branching, the length of the hydrocarbon chain, and the number of functional groups (1–5). Acid- or base-catalyzed hydrolysis of complex lipids yields the free FA. These long- or short-chain acids are generally referred to by their common names, which in most cases reflect their sources. Natural FA can be saturated, unsaturated, or branched. The saturated (branched) acids have higher m.p. than unsaturated acids of corresponding size (6).

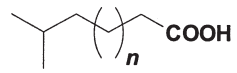
Branched fatty (carboxylic) acids and/or their derivatives, e.g., amides, alcohols, and aldehydes, whose ends are remote from the carboxylic (or $–NH_2$, -OH, and –CHO) group, as shown in Figure 1, are usually described by the terms *iso*-, *anteiso*-, or *neo*-. The sterically hindered structures of neo acids provide them with certain performance advantages over the unhindered FA. Natural biogenic neo acids and/or alkanes containing a tertiary butyl group(s) are produced by different organisms (bacteria, fungi, plants, marine invertebrates, and animals) for a specific purpose, usually involving the survival of the species. Research into the natural occurrence of neo acids is relatively new with much attention in the past focusing on the pharmaceutical impact of synthetic ones. Neo (saturated tertiary and/or trialkylacetic) acids are synthetically produced and have been available since the early 1960s (6,7).

Discussions of the physical and chemical properties of neo acids, their manufacture, economic aspects, storage and handling, toxicity, and applications have been reviewed (5,8–10).

Neo acids and/or their derivatives show different biological activities, including anticarcinogenic, antifungal, antibacterial, antimicrobial, and others (11).

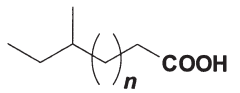
Neo alkanes are produced by soil cyanobacteria (12), marine algae (13), and some microorganisms (14,15). They have been isolated from plant species and also have been discovered in ancient sediments and oils (16,17). Aromatic and related compounds containing a tertiary butyl group(s), which are produced by many plants, are of considerable interest for chemistry, nutrition, commerce, and human health (18) and are considered in this review..

This paper is a comprehensive survey of neo acids, neo alkanes, and their analogs and derivatives that are deemed as naturally occurring. Also, this is the first article to review natural neo fatty (carboxylic) acids, neo alkanes, and other metabolites containing a tertiary butyl group(s) [or *tert*-butyl unit(s)].



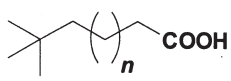
iso-fatty acids

n = -1 to 38



anteiso-fatty acids

n = 2 to 30



neo-fatty acids

n = -1 to 13

FIG. 1. The *iso*-, *anteiso*-, and *neo*-fatty (carboxylic) acids isolated from living organisms.

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Abbreviations: 6FP, 6-formylpterin; CDH, choline dehydrogenase; DC-A, discodermin A; EC_{50}, concentration having an effect on 50% of the population; LC_{50}, concentration having a lethal effect on 50% of the population; PAF, platelet-activating factor.

OCCURRENCE OF NEO ACIDS AND DERIVATIVES IN NATURE

Pivalic acid (**1**), a simple carboxylic acid with methyl side chains, is usually used in the form of its chloride as an intermediate in the manufacture of pharmaceuticals (ampicillin, amoxycillin, cephalosporins), pesticides, and organic peroxide compounds (19). Other names for pivalic acid include: 2,2-dimethyl propionic acid, dimethylpropionic acid, α,α-dimethyl propionic acid, *tert*-pentanoic acid, neopentanoic acid, trimethylacetic acid, and versatic acid 5.

Pivalic acid was detected in snake fruit (*Salacca edulis*) (20); white- and yellow-fleshed peaches, peento peach, and nectarine (21); in the phenolic fractions from Koshu and Zenkoji grapes (22); in *Capsicum annuum* var. *annuum* fruits (23); in commercial plain sufu [fermented soybean (*Glycine max*) curds] (24); in tobacco leaf material (25); in the volatile, neutral and acidic constituents of Virginia tobacco (26); and in the volatile flavor of a wheat flour and butter mixture, and roux cooked to 100°C (27). Pivalic acid also was detected in ripe and unripe golden empress melon (*Cucumis melon*) (28) and *Michelia alba* (29), and in the exotic fruit *Spondias mombin* from French Polynesia (30).

It also was detected in the Japanese fish sauce Ishiru (31), in three brands of Philippine fish sauces (patis) (32), in fish sauce (33), and in volatile flavor components in snow crab cooker effluent and effluent concentrate (34).

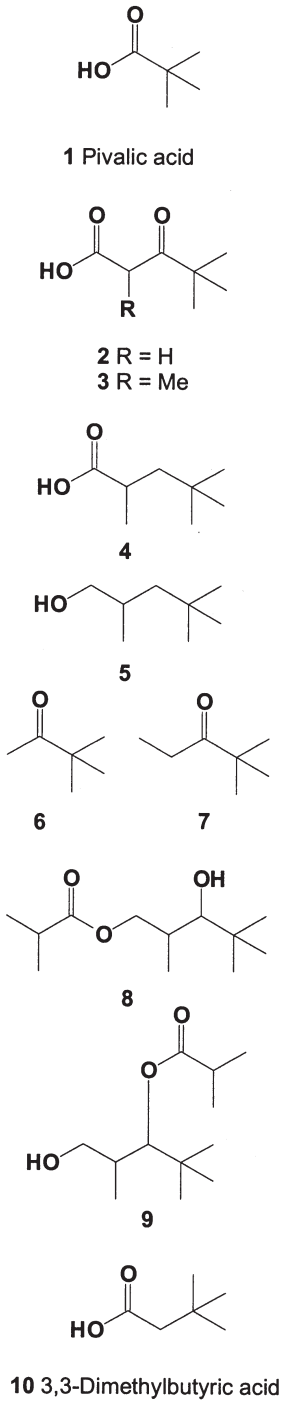
Numerous bacterial strains able to degrade pivalic acid have been isolated, including members of families Rhodocyclaceae, Comamonadaceae, and Oxalobacteraceae; and two pathways have been proposed (35). After activation with a CoA-forming ligase, a pivalyl-CoA mutase is considered to isomerize the quaternary compound. In contrast to the originally proposed pathway, recent studies showed that 3-methylbutyryl-CoA is the product. In an alternative pathway, strain PIV-34-1, a member of Oxalobacteraceae that is closely related to genus *Herbaspirillum*, was proposed to degrade pivalate *via* an anaerobic oxidation of the methyl group, with dimethylmalonate as the initial product (35).

Some metabolites of pivalic acid—pivaloylacetic acid (**2**,**3**), methyl-neopentylacetic acid (**4**), 2,4,4-trimethyl-1-pentanol (**5**), and 4,4-dimethyl-3-pentanone (**6**)—were isolated from extract of *Mycobacterium austroafricanum* IFP 2173 during investigation of the catabolic pathway of isooctane (36).

2,2-Dimethyl-3-pentanone (**7**) was produced by the toxic fungi *Stachybotrys chartarum*, *Aspergillus* spp., and *Penicillium* spp. (37). A mixture of five fungi—*Aspergillus versicolor, Fusarium culmorum, Penicillium chrysogenum, Ulocladium botrytis,* and *Wallemia sebi*—produced many volatile organic compounds, including two neo metabolites, (**8**,**9**) (38).

Simple neohexanoic (3,3-dimethylbutanoic) acid (**10**) was isolated from volatile constituents of *Hemerocallis citrina* (daylily, Liliaceae) (39); and it was also detected in *Ferula gummosa* essential oil (40). Traditional herbal medicines based on *F. gummosa* have been used for treatment of intestinal disorders in Iran, and *F. gummosa* essential oil showed spasmolytic activity. Neohexanoic acid was detected in Saint-Maure soft cheeses obtained from goat's milk (41).

Pivalic acid was found in samples of the Cl-Na-Ca type stratal waters of petroleum deposits (Neftechala, Azerbaidzhan) (42). Pivalic acid and a homologous series of low M.W. monocarboxylic acids (C_2 to C_{12}) and γ-lactones (C_5 to C_{12}) were detected in Miocene to Pliocene sediments of Shinjo Basin (43). More than 50 monocarboxylic acids, including pivalic acid, neohexanoic acids, and neopentyl 3,3-dimethylbutanoate (**12**), were detected in the water-soluble organic com-



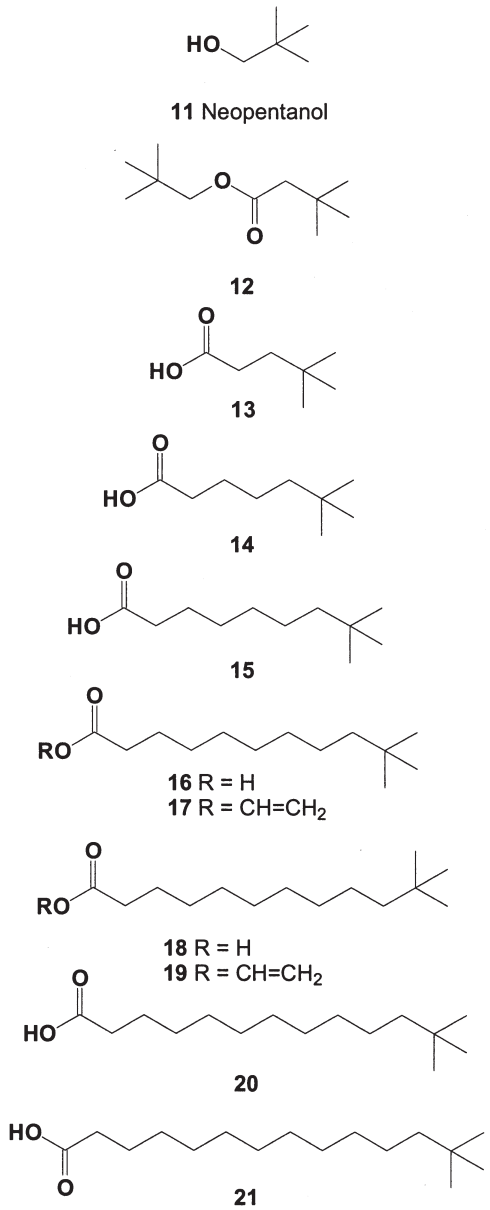
pounds of Murchison and EET96029.20 carbonaceous meteorites (44). The distribution of low M.W. monocarboxylic acids was studied in three CM2 Asuka carbonaceous chondrites (A-881280, A-881334, and A-881458) that were recovered from Antarctica by the 29th Japanese Antarctic Research Expedition in 1988. More than 30 monocarboxylic acids were isolated, including aliphatic and aromatic acids with various structural isomers, including neohexanoic acid (45).

Echinacea, the purple coneflower, is the best known and researched herb for volatile constituents, and neopentanol (**11**) was detected in three *Echinacea* species (46). Pivalic acid, neohexanoic acid, and neopentanol were isolated from sheep wool extracts, and it was shown that these compounds are hair growth stimulants (47), and from *Inula viscosa* extract (48). Sweet olive (*Osmanthus fragrans*) is a flower native to China that is valued for its delicate fragrance, and neopentanol was isolated from the top note of *O. fragrans* (49). Volatile components of pine nuts were recovered by simultaneous distillation and extraction after Soxhlet extraction. One hundred eighteen components, including 26 hydrocarbons, 17 esters, 16 aldehydes, 12 ketones, 31 alcohols, 11 bases, 2 acids and neopentanol were identified (50).

The neo FA having structures **13**–**16** and **20** have been isolated as minor components from the glycolipids fraction of the freshwater sponges, *Ephydatia syriaca*, *Nudospongilla* sp., and *Cortispongilla barroisi* (51). Neononanoic (**16**) and neodecanoic acids (**18**) and its ethenyl esters (**17** and **19**), respectively, were found in lubricating oils of animal and vegetable origin (52-54).

Neohexadecanoic acid (13,13-dimethyltetradecanoic acid) (**21**) was isolated from both wood and bast (inner bark) of *Daphne bholua* (also known as "Jacqueline Postill," and a bush used in Nepal for paper manufacture) along with other FA. Bast contained more unsaturated FA than wood (55). The composition of higher FA and diterpenic acid mixtures was determined by HPLC in fir and cedar chlorophyll-carotene paste from needles, in fir balsamic paste from needles, and in fir bark lipid constituents. The main unsaturated acids found in the samples were linoleic, oleic, and linolenic; the main saturated acids were palmitic acid and 14-methylhexadecanoic acid, and among branched FA 13,13-dimethyltetradecanoic acid (**21**) was detected. The mixtures of diterpenic acids contained mainly abietic, dehydroabietic, neoabietic, levopimaric, palustric, isopimaric, sandaracopimaric, and pimaric acids (56). The total content of FA and rosin acids in pine needles was 2.2–2.3%, of which rosin acids account for 92–93%. The content of FA in needles decreases and that of rosin acids increases with increasing age of needles. Among 38 rosin acids and FA isolated from pine needles, 13,13-dimethyltetradecanoic, palmitic, stearic monomethyl pinifolate, and neoabietic acid were dominant compounds. (57). FA mixtures isolated from the balsams of *Picea excelsa* and *P. sibirica* contain large amounts 7-hexadecenoic, 4,7,10-hexadecatrienoic, 6,9,12,15-octadecatetraenoic, and 11,14-eicosadienoic acids. All studied Soviet balsams and rosins of different conifers contained 13,13-dimethyltetradecanoic acid (**21**) as a minor compound (58). The composition of FA of Bulgarian extractive colophony and pine resin from *Pinus silvestris* and *P. nigra* was studied, and some branched acids—11-methyl-dodecanoic, 12-methyltridecanoic, 13-methyltridecanoic, 12-methyltetradecanoic, 13,13-dimethyltetradecanoic (**21**), and 14-methylpentadecanoic—were detected (59). Neohexadecanoic acid was also isolated from the mixture of saturated FA in tall oils from the Segezha and Kotlass pulp mills (60).

Neopalmitic acid (**21**) was found in some marine invertebrates. Phospholipids and FA of the shell, chitin, and chitosan of the crab *Paralitodes camtshatica* were studied (61). PC, PE, and PS were found in the shell, and PC in chitin, and diphosphatidylglycerols in chitosan. The shell had more FA than did chitin and chitosan, and the most prominent FA in the shell were 9-octadecenoic acid (22%), *iso*-palmitic acid (10%), 9,12-octadecadienoic acid (10%), and 6-hexadecenoic acid. The



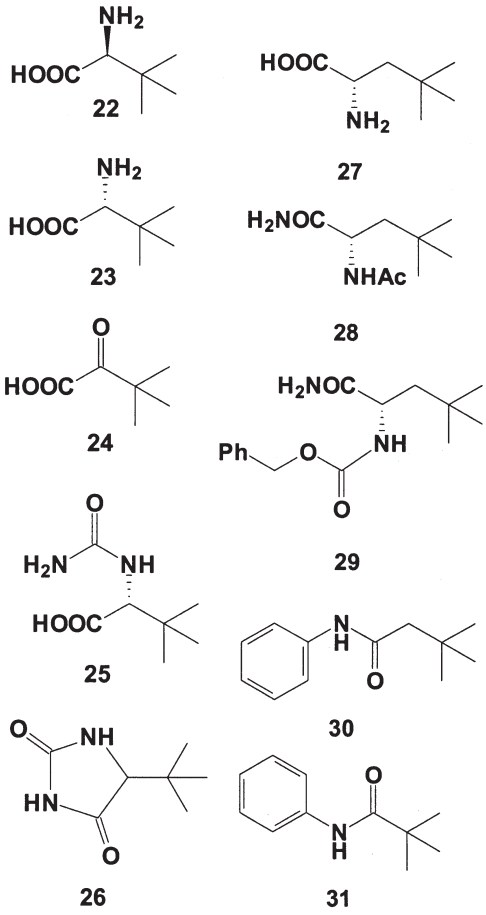
most prominent FA in the chitin were neopalmitic acid (**21**) (22%), 9-octadecenoic acid (19%), myristic acid (12%), and 6-hexadecenoic acid (12%). The most prominent FA in chitosan were 9,12-octadecadienoic acid (18%), palmitic acid (16%), and nondecylic acid (9%) (61). Neopalmitic acid (**21**) was also found in the crayfish, *Astacus astacus* (62).

(2*R*)-2-Amino-3,3-dimethylbutanoic acid (**22**) was produced by 54 soil bacteria taken on Hat Yai campus of Prince of Songkla University (Thailand) when grown at 30 and 45°C (63). (2*S*)-2-Amino-3,3-dimethylbutanoic acid (**23**) and 2-oxo-3,3-dimethylbutanoic acid (**24**) were obtained by transamination with transaminase-producing yeast and bacteria such as *Arthrobacter* sp., *Brevibacterium* sp., *Brevibacterium lineus*, *Brevundimonas* sp., *Candida* sp., *Candida utilis*, *Corynebacterium ammoniagenes*, *Cryptococcus* sp., *Cryptococcus flavus*, *Flavobacterium heparinum*, *Hansenula* sp., *Hansenula jadinii*, *Issatchenkia orientalis*, *Micrococcus* sp., *Micrococcus luteus*, *Nocardioides simplex*, *Pedobacter heparinus*, *Pichia* sp., *Schizosaccharomyces* sp., and *Schizosaccharomyces pombe* (64).

A novel *Pseudomonas putida* strain produced optically pure chiral amino acids and their derivatives (**23**) including N-(aminocarbonyl)-3-methyl-L-valine (**25**) and 5-(1,1-dimethylethyl)-2,4-imidazolidinedione (**26**) (65). Microbial peptide amidases obtained from soil microorganisms in a double screening were used for the production of peptides and neo N-terminal-protected amino acids and racemate splitting of N-protected amino acid amides, D-amino acids, and Nα-protected D-amino acid amides (**27**–**29**) (66). Two amides (**30**) and (**31**) were found in an extract of the root bark of *Viburnum prunifolium* (67).

The marine bacterial community (EM4, including the genera *Marinobacter, Bacillus,* and *Erwinia*) produced several neo compounds—2,4,4-trimethyl-2-penten-3-ol (**32**), 2,4,4,6,6-pentamethylheptanoic acid (**33**), 2,4,4,6,6-pentamethylheptanoic acid (**34**), 2,4,4,6,6-pentamethyl-3-oxo-heptanoic acid (**35**), 2,4,4,6,6-pentamethyl-5-oxo-heptanoic acid (**36**), 2,4,4-trimethylpentanoic acid-1,1-dimethylethyl ester (**37**), and 2,2,4,6,6,8,8-heptamethyl-3-nonanone (**38**)—by direct oxidation of 2,2,4,4,6,8,8-heptamethylnonane (68). The volatile oils isolated by steam distillation from the leaves, stems and flowers of *Paederia foetida* (Malaysia) led to a new neo kyowanoic N (3,5,5-trimethylcaproic, **39**) acid (69).

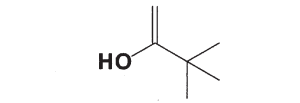
Pivaloylcarnitine (**40**), pivaloylglycine (**41**), and free pivalic acid (**1**) were present in human urine samples after administration of S-1108 (70). Metabolism of pivaloylcarnitine and pivaloyl-CoA, as well as carnitine homeostasis in man were studied and reviewed (71). *tert*-Valeraldehyde (or pivalaldehyde, **42**) was isolated from among 39 alcohols, 31 aldehydes, 28 ketones, 17 esters, 7 lactones, 4 phenols, 3 furans and furanones, 29 hydrocarbons, and 14 other compounds from black tea (72); it was also present in cigar smoke (73). Raw and smoked black bream (*Brama raii*) and rainbow trout (*Oncorhynchus mykiss*) contain some neo compounds (**43**–**45**) (74). The volatile oil of *Lysimachia foenum-graecum*, grown in Jinxiu County of Guangxi Zhuang Autonomous Region, contains compound **45** (75).



Dimbunol (**46**) was among 85 constituents of the essential oil of fresh flowers of *Elaeagnus angustifolia* (76). Dimbunol is a competitive inhibitor of choline dehydrogenase (CDH)—more so than ethylcholine mustard aziridinium ion—and is an effective irreversible inhibitor of both CDH and choline transport in mice (77). 2,2-Dimethylpropionamide (**47**) was produced by *Pseudomonas putida* and by a strain of *Agrobacterium radiobacter* that was capable of converting nitriles to amides (78,79). 2-Methyl-2-propanol (**48**) was produced by seven *Pseudomonas* strains belonging to six different species: *Ps. brenneri*, *Ps. libanensis*, *Ps. graminis*, *Ps. lundensis*, *Ps. putida*, and *Ps. rhodesiae* (80). Rare 2,2-diethylbutyric acid (**49**), also known as NSC 407528 and NSC 938, was found in a natural anaerobic ecosystem (81).

During genetic engineering of *Streptomyces hygroscopicus* and other microorganisms for production of polyketides and other natural products, an unusual neo acid, 4-*tert*-butylcyclohexanecarboxylic acid (**50**) (also known as NSC 176105, NSC 176107, and NSC 48094), was isolated (82). Derivatives of bicyclo[3.1.0]hexan-2-one (**51**) were detected in essential oil of *Paulownia tomentosa* flowers (83); compounds **51** and **52** were found among volatile components of the marine red alga *Corallina officinalis* (84), and compound **51** has been isolated from the roots of *Aristolochia asclepiadifolia* (85).

An anticoagulant coumarin derivative, named pivalylindandione (**53**) (other names: pindone, pival, or pivalyl), was iso-



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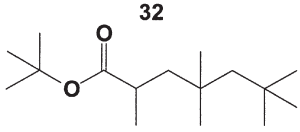

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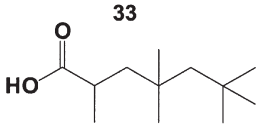

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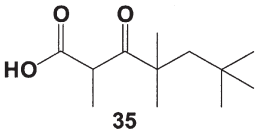

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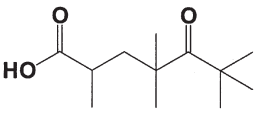

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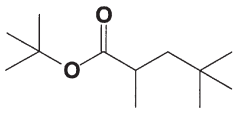

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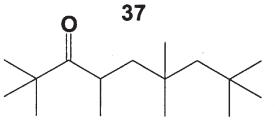

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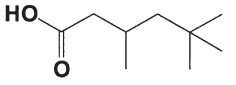
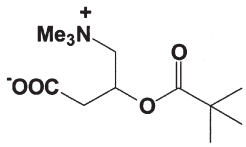
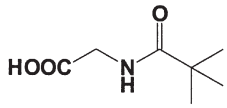


39 Kyowanoic acid N



40 Pivaloylcarnitine



41 Pivaloylglycine

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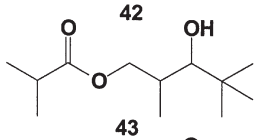

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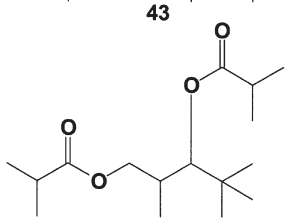

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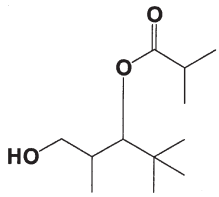

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lated from biological and bait materials (e.g., slugs, snails) more than 50 years ago (86). More recently, it has been shown that pivalylindandione has insecticidal and blood coagulation properties (87–91).

Two *cis*- and *trans*-isomers derivatives of cyclohexanol (**54, 55**) were obtained by transformations of four alicyclic ketones of an axenic strain of *Chlorella pyrenoidosa* (92).

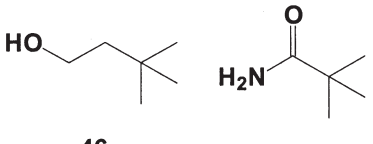
Laingolide (**56**), a novel 15-membered macrolide containing a lactam group, was isolated from the cyanobacterium *Lyngbya bouillonii*, collected on Laing Island (Papua-New Guinea) (93). Two macrolide derivatives, laingolide A (**57**) and madangolide (**58**), have been from obtained from the same organism (94).

Investigation of the blue-ring sea hare *Stylocheilus longicauda* led to the isolation of two chlorinated complex proline esters, makalika ester (**59**) and makalikone ester (**60**) (95).

Several biologically active metabolites with condensed ring systems and containing a *tert*-butyl unit belonging to sesquiterpenes (**61**–**68**) have been obtained from the leaves of *Ginkgo biloba* (96–99). Ginkgo is a traditional herb that has been used for disease treatment for more than 2,500 years (100). Extracts from its leaves were used in ancient China, whereas in the Western world they have been used only since the 1960s, when it became technically possible and feasible to isolate the essential substances of *Ginkgo biloba* (101).

The terpene trilactones of *Ginkgo biloba* leaves, containing mainly ginkgolides (A,B,C,J,M,K,L, **61**–**67**, respectively) and bilobalide (**68**), are selective platelet-activating factor (PAF) antagonists and have neuroprotective effects. Compound **62** especially has the greatest potentcy among these terpene trilactones and is now used as the PAF antagonist for the prevention and treatment of thrombus, illness of blood vessels of heart and brain, arrhythmia, asthma, bronchitis, senile dementia, and allergic reaction (102–104).

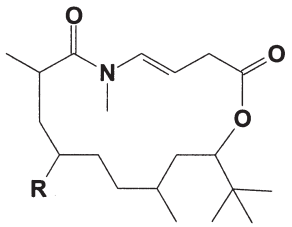
Ypaoamide (*Z*) (**69**), a new herbivore antifeedant metabolite, was isolated from the extract of a mixed cyanobacterial assemblage that was composed of *Schizothrix calcicola* and *Lyngbya majuscule* (105). Ypaoamide is a structurally novel cyanobacterial metabolite.



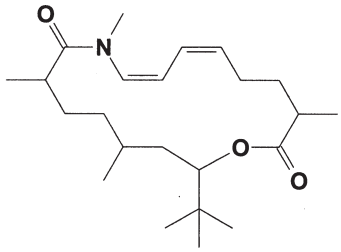
46 47

48 49

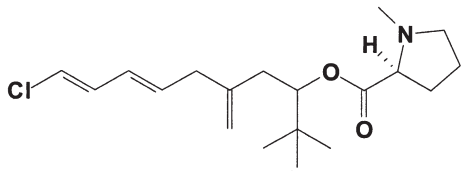
Ypaoamide (*E*) (**70**) was isolated from a cyanobacterial bloom and a massive die-off of juvenile rabbitfishes (*Siganus argenteus* and *S. spinus*). The microbial assemblage was composed primarily of the marine cyanobacterium *Schizothrix calcicola* with sparsely distributed strands of *Lyngbya majuscula*. Palatability of the crude organic extract of this mixed cyanobacterial assemblage was evaluated in feeding assays using the parrotfish *Scarus schlegeli* and the sea urchin *Echinometra mathaei* in outdoor tanks on Guam. Bioassay-guided fractionation resulted in the isolation of ypaoamide B, a new, broadly acting feeding deterrent compound (106,107).



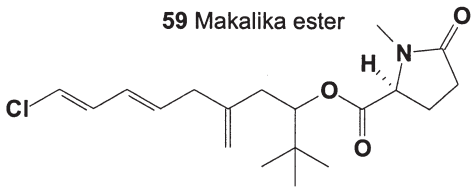
56 Laingolide, R = Me
57 Laingolide A, R = H



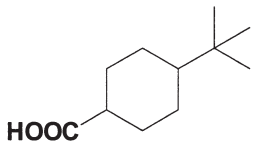
58 Madangolide



59 Makalika ester



60 Makalikone ester



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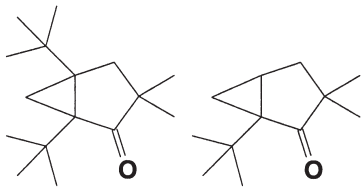
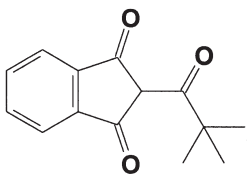


51 **52**



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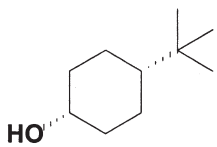

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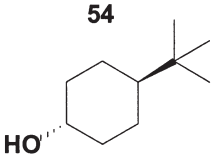
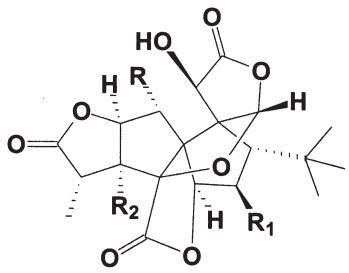


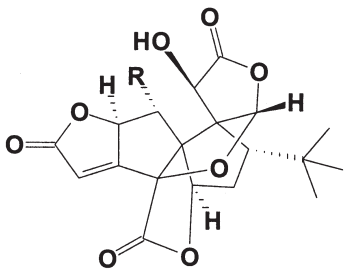
55

Teleocidins are potent tumor promoters, having a nine-membered lactam structure. Teleocidins and their small-molecular-sized active congeners (indolactams) are known to exist in an equilateral position between at least two conformational states, the twist and the sofa form. Three teleocidin derivatives (**71**–**73**) with a *tert*-butyl unit are produced by the actinomycete, *Streptoverticillium blastmyceticum* NA34-17 (108–111).

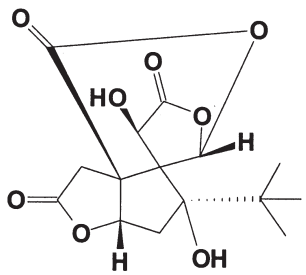
The antibiotics bottromycin A2 (**74**) and B (**75**) are produced by *Streptomyces* sp. strain No. 3668-L2 (112,113), *Kitasatoa purpurea* strain KA-281 (114), and *Micromonospora chalcea* strain FERM-P 1823 (115). Bottromycins showed similar antibacterial spectra, except that bottromycin A was 3–4 times stronger than bottromycin B. They were effective against six antibiotic-resistant strains of *Staphylococcus aureus*, *Streptococcus pyogenes, Micrococcus flavus, Bacillus subtilis, B. cereus, B. megaterium, B. anthracis, Corynebacterium xerosis*, and *Mycobacterium phlei* in concentrations of 0.03–3 μg/mL.



61 Ginkgolide A, R,R_1, = H, R_2 = OH
62 Ginkgolide B, R,R_2 = OH, R_1 = H
63 Ginkgolide C, R,R_1,R_2 = OH
64 Ginkgolide J, R = H, R_1,R_2 = OH
65 Ginkgolide M, R,R_1 = OH, R_2 = H



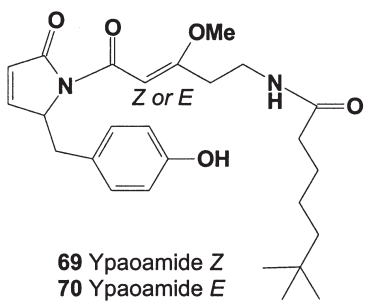
66 Ginkgolide K, R = OH
67 Ginkgolide L, R = H



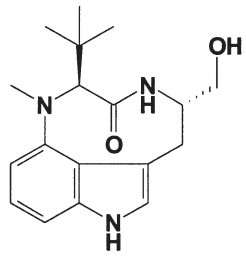
68 Bilobalide

Lesser activity was exhibited against *Klebsiella pneumoniae*, three strains of *Escherichia coli, Salmonella typhosa*, and *Shigella dysenteriae* (112).

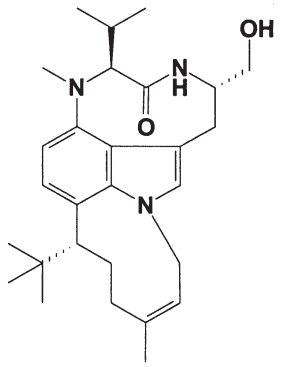
The ichthyotoxic cyclic lipopeptide antillatoxin A (**76**) was isolated from the cyanobacterium *Lyngbya majuscula* (116,117).



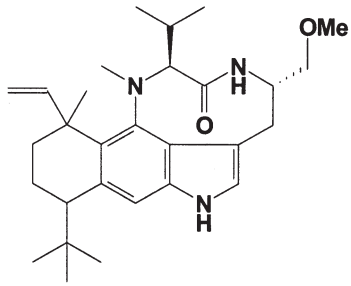
69 Ypaoamide *Z*
70 Ypaoamide *E*



71 Indolactam TL



72 Blastmycetin E

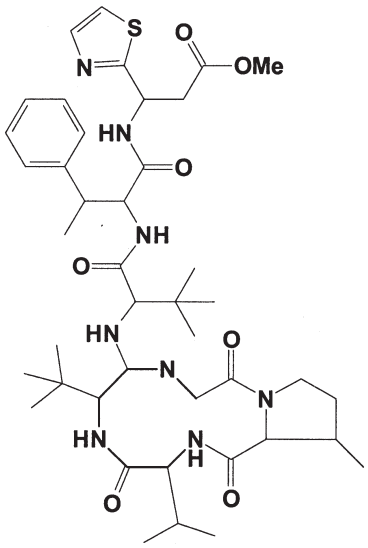


73 Olivoretine E

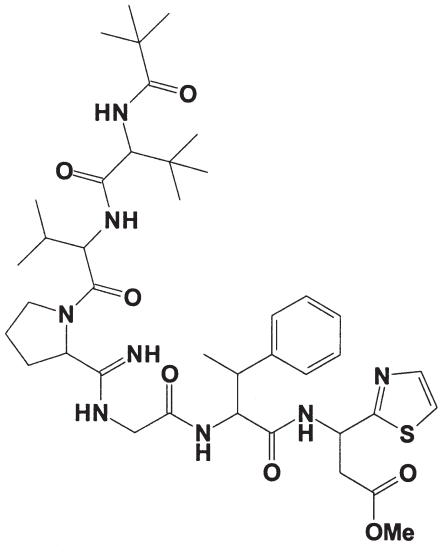
Goldfish toxicity was used to direct the fractionation of a chemical-rich extract of *Lyngbya majuscula* from Curaçao, resulting in the isolation of a metabolite, (**76**), as an exceptionally potent ichthyotoxin (LD_{50} = 0.05 μg/mL).

Bioassay-guided fractionation of organic extracts from two *Lyngbya majuscula* collections led to the isolation of a new secondary metabolite, antillatoxin B (**77**), an unusual N-methyl homophenylalanine analog of the potent neurotoxin antillatoxin. Antillatoxin B exhibited significant sodium channel-activating (EC_{50} = 1.77 μM, where EC_{50} refers to that concentration having an effect on 50% of the population) and ichthyotoxic (LC_{50} = 1 μM, where LC_{50} is that concentration having a lethal effect on 50% of the population) properties (118,119). The potent cytotoxic agent hemiasterlin (**78**) was identified from the sponge *Hemiasterella minor* (120). Antimiotic peptides, hemiasterlin (**79**), hemiasterlin methyl ester (**80**), dihydrohemiasterlin (**81**), and criamides A (**82**) and B (**83**) were isolated from Indian Ocean sponge *Cymbastela* sp. (121,122).

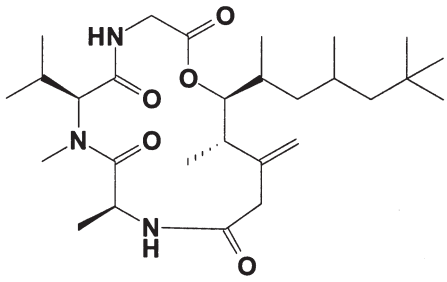
A reinvestigation of the sponge *Auletta* sp. yielded the novel and known cytotoxic compounds, milnamide A, C, and D (**84**–**86**), hemiasterlin, jasplakinolide, and geodiamolides A, D,



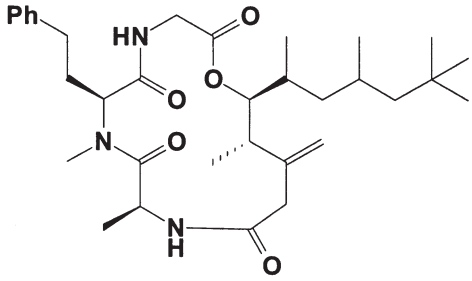
74 Bottromycin A_2



75 Bottromycin B



76 Antillatoxin A



77 Antillatoxin B

E, and G, and milnamide B (known as hemiasterlin, **78**) (123). Milnamides A and D were also found in methanolic extract of the marine sponge *Cymbastela* sp. (124).

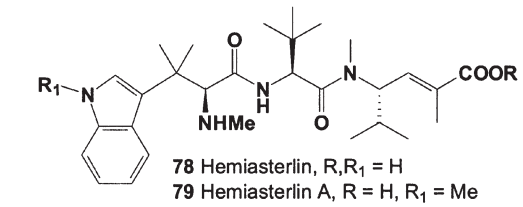
A depsipeptide, polydiscamide A (**87**), composed of 13 amino acids including a novel amino acid 3-methylisoleucine, was isolated from a Caribbean sponge of the genus *Discodermia* (125). This compound inhibits the *in vitro* proliferation of the cultured human lung cancer A549 cell line. Microspinosamide (**88**), a new cyclic depsipeptide incorporating 13 amino acid residues, was isolated from extracts of an Indonesian collection of the marine sponge *Sidonops microspinosa*. The tridecapeptide (**88**) incorporates numerous uncommon amino acids, and it is the first naturally occurring peptide to contain a β-hydroxy-*p*-bromophenylalanine residue. Compound **88** inhibited the cytopathic effect of HIV-1 infection in an XTT (kit assay)-based *in vitro* assay with an EC_{50} value of 0.2 μg/mL (126).

The unique polytheonamides A (**89**) and B (**90**) are highly cytotoxic polypeptides with 48 amino acid residues. They have been isolated from the marine sponge, *Theonella swinhoei*. Elucidation of the structure of polytheonamide B resulted in an unprecedented polypeptide structure: an N-terminal glycine blocked with a 5,5-dimethyl-2-oxo-hexanoyl group, the presence of eight *tert*-leucine, three β-hydroxyvaline, six γ-N-methylasparagine, two γ-N-methyl-β-hydroxyasparagine, and β,β-dimethyl-methionine sulfoxide residues. More significantly, there is a sequence of alternating D- and L-amino acids. Polytheonamide A is an epimer of polytheonamide B, differing only in the stereochemistry of the sulfoxide of the 44th residue (127). Polytheonamides A and B are quite unusual in that one peptide molecule contains nine amino acids with *tert*-butyl units (!).

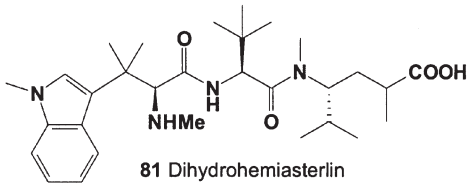
Three tetradecapeptides named halicylindramides A–C were isolated from the Japanese marine sponge *Halichondria cylindrata*; halicylindramide B (**91**) and C (**92**) contain *tert*-butyl units. In these tetradecapeptides the N-terminus is blocked by a formyl group and the C-terminus is lactonized with a threonine residue. Halicylindramides A–C have antifungal activity against *Mortierella ramanniana* and cytotoxic activities against P388 murine leukemia cells (128).

Two collections of the marine cyanobacterium *Lyngbya* sp. from Guam and Palau both afforded the potent cytotoxins apratoxin A (**93**) and B (**94**) and E-dehydroapratoxin A (**95**). According to SAR studies on this novel family of depsipeptides, these compounds showed toxic activity against some yeast species. The key structural elements responsible for the cytotoxicity of these toxins were identifiedAll analogs displayed weaker cytotoxicity than compound **93**, but to different extents (129).

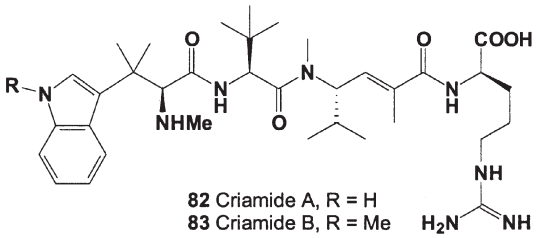
The structure of the antimicrobial peptide discodermin A (**96**) isolated from the marine sponge *Discodermia kiiensis*



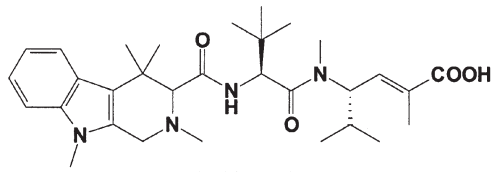
78 Hemiasterlin, R,R_1 = H
79 Hemiasterlin A, R = H, R_1 = Me
80 R,R_1 = Me



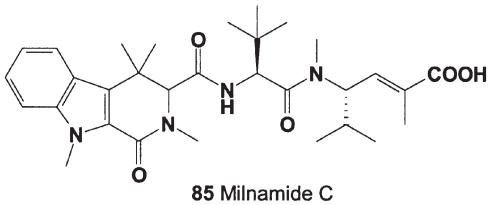
81 Dihydrohemiasterlin



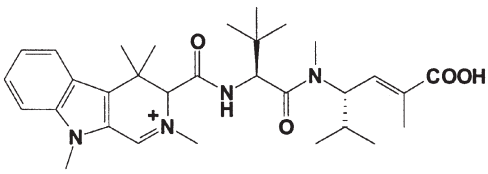
82 Criamide A, R = H
83 Criamide B, R = Me



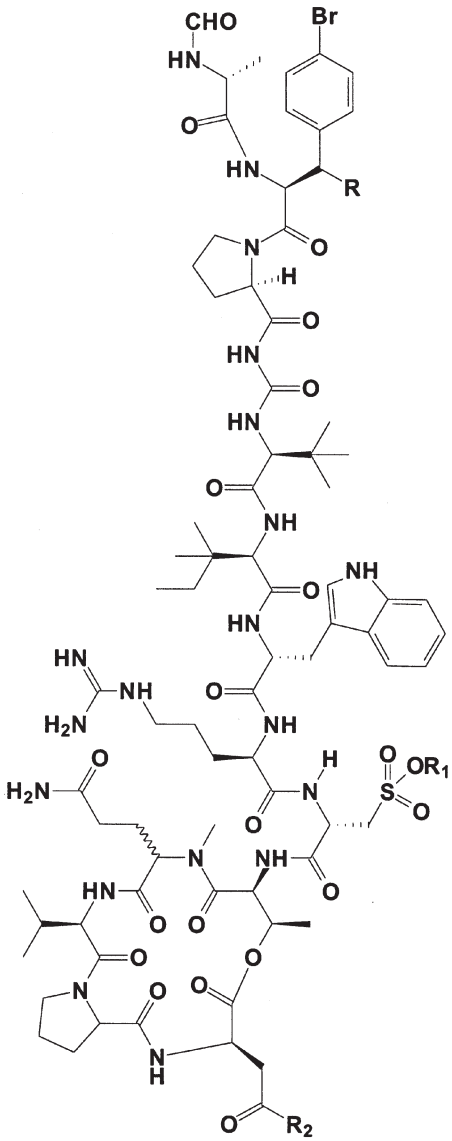
84 Milnamide A



85 Milnamide C



86 Milnamide D

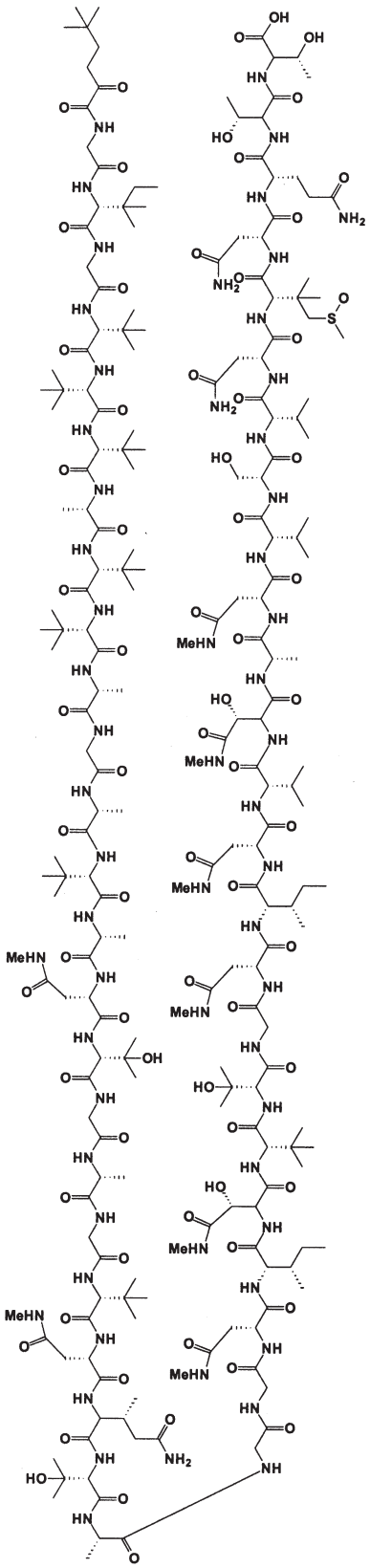


87 Polydiscamide A,
R,R_2 = OH, R_1 = H
88 Microspinosamide,
R = H, R_1 = Na, R_2 = NH_2

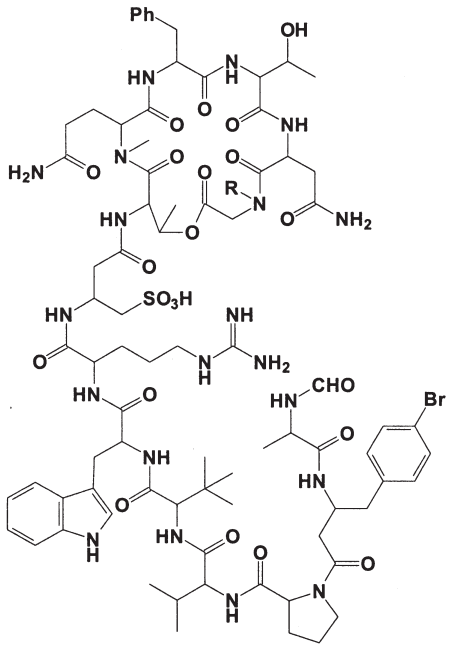
were elucidated in 1984 (130). Ten years later, the known discodermins A–D were isolated from same sponge together with a cytotoxic and antimicrobial tetradecapeptide discodermin E (**97**) (131) and three novel tetradecapeptides, named F (**98**), G (**99**), and H (**100**) (132). The effects of discodermin A (DC-A), a marine bioactive peptide extracted from the sea sponge *D. kiiensis*, on the vascular smooth muscle cells and tissues were reported (133). Analysis with a confocal laser microscope showed that DC-A (0.1–30 μM) permeabilized the plasma membrane of A10 cells to the nonpermeable fluorescent agents ethidium homodimer-1 and calcein in a concentration-dependent manner. In vascular tissue treated with 30 μM DC-A, addition of a micromolar concentration of Ca^{2+} evoked a sustained contraction in the presence of ATP, suggesting that DC-A increased the permeability of the membrane to Ca^{2+} and ATP. These results suggested that DC-A had a permeabilizing effect on the plasma membrane, possibly by interacting with plasma membrane phospholipids with its six successive hydrophobic amino acid residues at N-terminal.

Several steroids containing a tertiary butyl group have been isolated from plants, marine algae, and invertebrates.

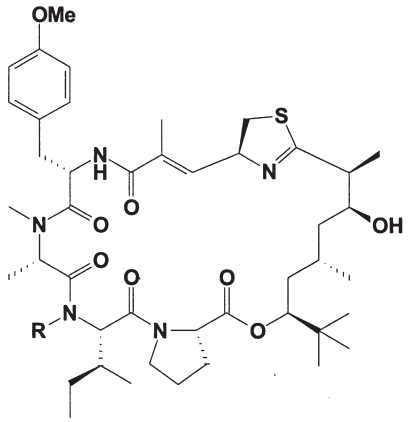
Wallenone (**101**), a C_{32} tirucallane-type triterpene, was isolated from the leaves of *Gyrinops wala* (Thymelaeaceae, small tree grown on Ceylon) (134). More recently, the same compound was isolated from the ethyl acetate extract of the dried leaves of *Esenbeckia stephani* (Rutaceae) (135).



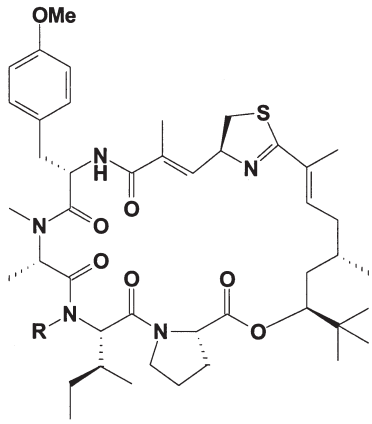
89, 90 Polytheonamides A and B



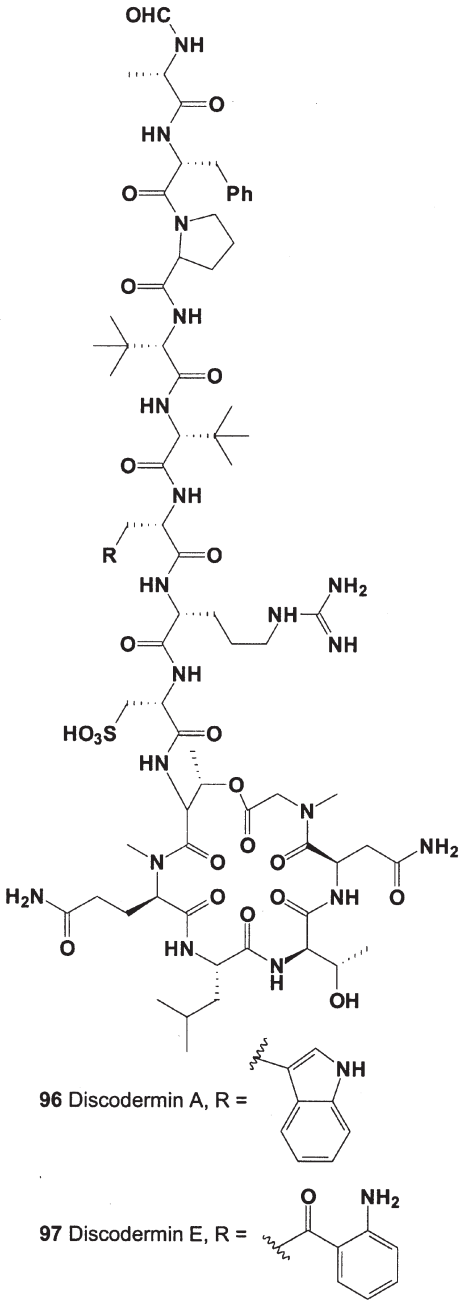
91 Halicylindramide B, R = H
92 Halicylindramide C, R = Me



93 Apratoxin A, R = Me
94 Apratoxin B, R = H

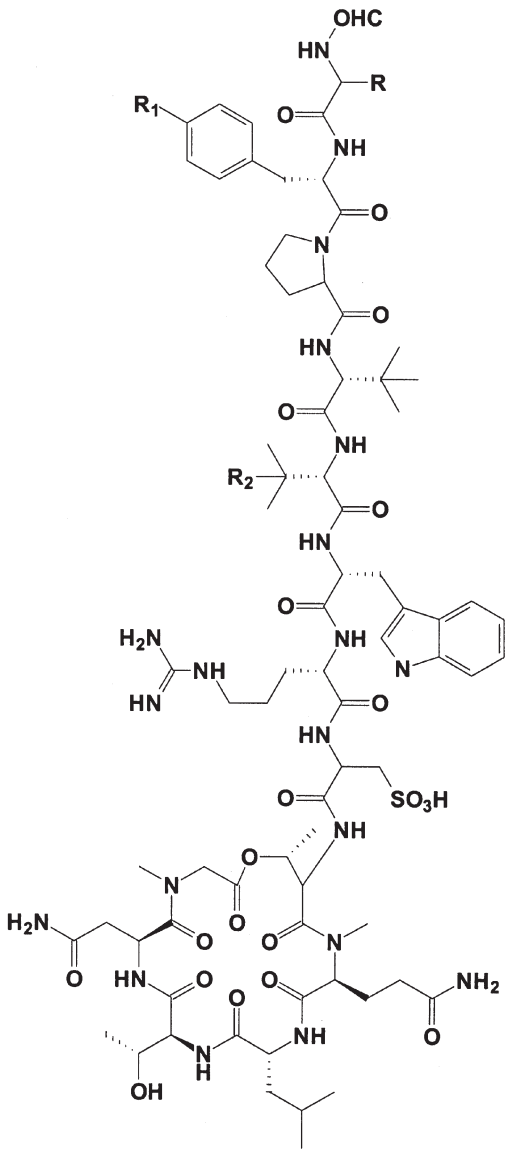


95 E-Dehydroapratoxin A



96 Discodermin A, R =

97 Discodermin E, R =

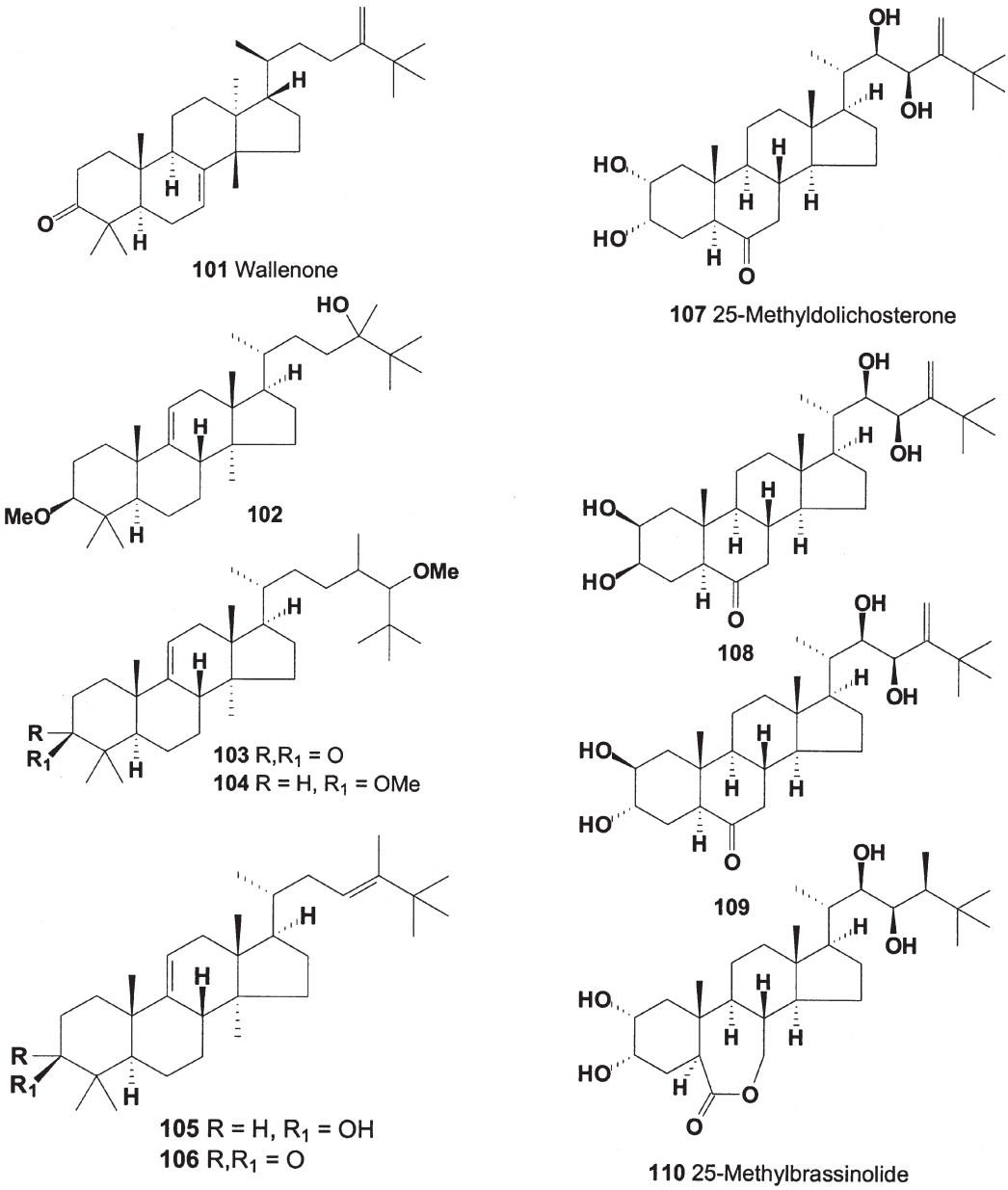


98 Discodermin F, R = R_1 = H, R_2 = Et
99 Discodermin G, R = R_2 = Me, R_1 = H
100 Discodermin H, R = H, R_1 = OH, R_2 = Me

Three triterpenoids, 3β-methoxy-24-methyl-lanost-9(11)-en-24-ol (**102**), 24ξ-methoxy-24,25-dimethyl-lanost-9(11)-en-3-one (**103**), and 3β,24ξ-dimethoxy-24,25-dimethyllanost-9(11)-ene (**104**), were isolated from *Neolitsea pulchella* (Lauraceae, Hong Kong, also known as Neolitsea) (136,137). Two triterpenoids, 24,25-dimethyl-9(11),23-lanostadienol (**105**) and 24,25-dimethyl-lanosta-9(11),23-dien-3-one (**106**), were obtained from the stems of *Quercus* spp. (*Q. bambusaefolia, Q. championi, Q. myrsinaefolia*) (138).

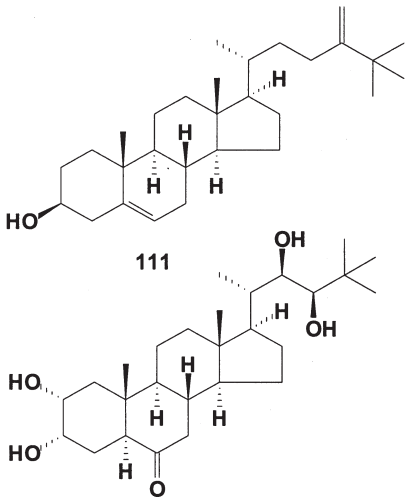
Several phytosterols with a tertiary butyl group, namely, 25-methyldolichosterone (**107**), 2,3-diepi-25-methyl-dolichosterone (**108**), 25-methyl-2-epidolichosterone (**109**), 25-methyl-brassinolide (**110**), 24-methylene-25-methyl-cholesterol (**111**), and brassinone (**112**) were identified in immature seed of *Phaseolus vulgaris*. 24-Methylene-25-methylcholesterol is considered closely correlated biogenetically with 25-methyl-dolichosterone (139–142). Compound (**110**) was a more potent plant-growth regulator than brassinolide itself (143).

In 1949, Bergmann and Feeney (144) discovered haliclonasterol (25-methyl-24ξ-ergosta 5,7,22- rien-3β-ol; **113**) in marine sponges belonging to genus *Haliclona*. They were studying the sterol composition of acetone extracts from the sponges *H. variabilis*, *H. permollis, H. coerulescens, H. viridis, H. rubens*, and *H. longleyi* and showed that haliclonasterol was present in all investigated species (144). Haliclonasterol was also detected in the marine green algae *Monostroma nitidum* and *Enteromorfa*



linza (145), and other species belonging to the phylum Chlorophyta (146,147). It also was found in other sponge species (148), and in three sea anemones, *Palythoa mammilosa*, *Zoanthus proteus*, and *Condylactis gigantean* (149). For the first time an axinyssasterol (**114**) and (3β,24*E*)-25-methyl-stigmasta-5,24(28)-dien-3-ol (**115**) were identified from a *Pseudoaxinyssa* species by Li and Djerassi (150). In the Australian sponge *Trachyopsis* sp., 64% of the total sterols was axinyssasterol (151). (3β,22*E*,24ξ)-28,28-Dimethyl-stigmasta-5,22,25-trien-3-ol acetate (**116**) and (3β,22E)-25-methyl-stigmasta-5,22-dien-3-ol (**117**) were obtained from an EtOH extract of the sponge *Halichondria* sp. (152,153), and compound **117** was also found in specimens of the sponge *Trachyopsis aplysinoides* from Sri Lanka inshore waters (154).

The antimicrobial halistanol (**118**) and its trisulfate (**119**) were isolated from the Okinawan sponge *Halichondria* cf. *moorei* more then 25 years ago (155). More recently, halistanol trisulfate was found in different sponge species (148,156–159). Halistanol trisulfate has a relatively low critical micelle concentration of 14.5 μM and strong hemolytic potency with an EC_{50} of 6.67 μM. As expected of a detergent, it inhibits the growth of Gram-positive but not Gram-negative bacteria (160). Halistanol sulfate E (**120**) was present in an extract of the marine sponge, *Epipolasis* sp. (161). Halistanol trisulfate and a new sterol sulfate, Sch 572423 (**121**), were detected in a marine sponge *Topsentia* sp.; both metabolites were identified as $P2Y_{12}$ inhibitors with IC_{50} of 0.48 and 2.2 μM, respectively (162). Some sponge steroids contain sulfate groups in positions 3α and 2β. This series of compounds includes compound **122**, a possible biogenetic precursor of halistanol sulfate (148). The tris-(2-aminoimidazolium) salt of halistanol sulfate (**123**) was isolated as an antimicrobial agent from a Japanese specimen of *Topsentia* sp. (163).

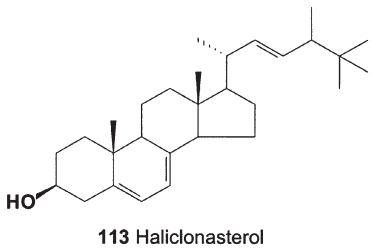


112 Brassinone

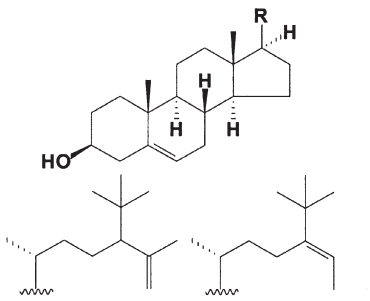
Several marine steroids with a tertiary butyl group were isolated as glycosides. Two novel triterpene glycosides, eryloside C (**124**) and eryloside D (**125**), from a sponge of the genus *Erylus*, collected at a depth of 500 m isouth of New Caledonia (an island off the northeast coast of Australia), were reported (164), and another glycoside termed eryloside E (**126**) was isolated from the marine sponge *E. goffrilleri* (165). The crude extract of sponge *E. nobilis* from Jaeju Island (Korea) exhibited moderate cytotoxicity (LC_{50} 317 μg/mL) against the human leukemia cell line K562. From this extract a new triterpenoid trisaccharide, designated erylosides I (**127**) and J (**128**), were obtained (166).

These compounds possessed C_{32} aglycone based on the carbon framework of lanostane. The sugar portions were proven to be branched assemblies of one unit each of L-arabinose, L-galactose, and 2-*N*-acetyl-L-glucosamine (**127**) or two units of L-arabinose and one unit of 2-*N*-acetyl-D-glucosamine (**128**) (166).

Some natural bioactive metabolites contain pivalic acid (**1**) in their structure. Bryostatins, a structurally novel family of marine macrolides, were first discovered by Pettit and co-workers in the bryozoan invertebrate *Bugula neritina* (167). These interesting anticarcinogenic compounds are also found in smaller quantities in the marine organisms *Lissodendoryx isod-*

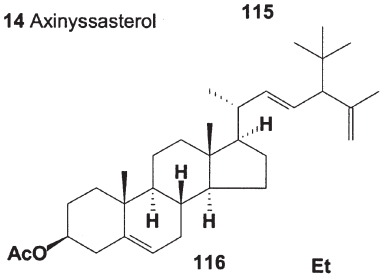


113 Haliclonasterol



114 Axinyssasterol

115



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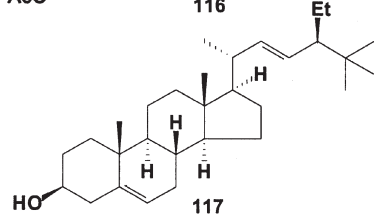

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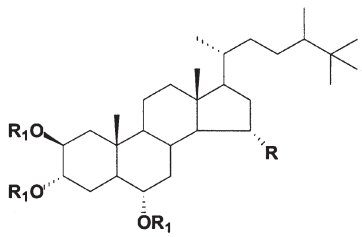
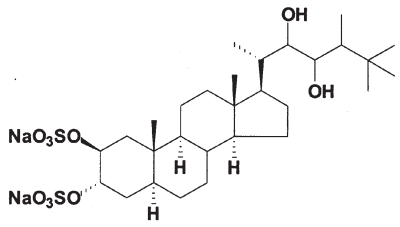
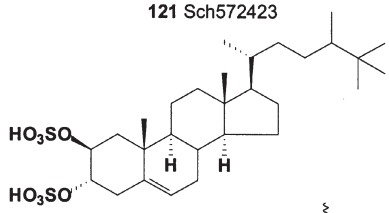


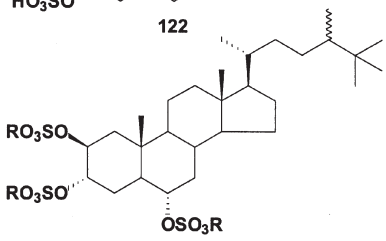
118 Halistanol, R,R_1 = H
119 Halistanol trisulfate, R = H, R_1 = SO_3H
120 Halistanol sulfate E, R = OH, R_1 = SO_3H

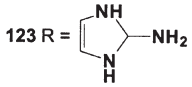


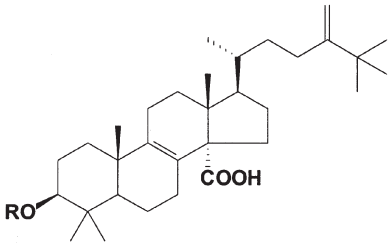
121 Sch572423



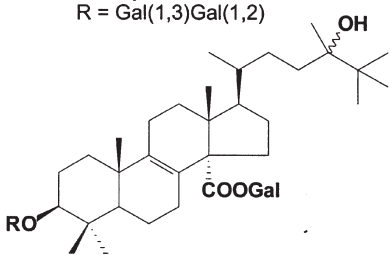
122



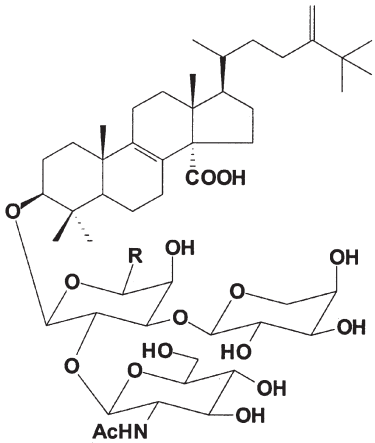




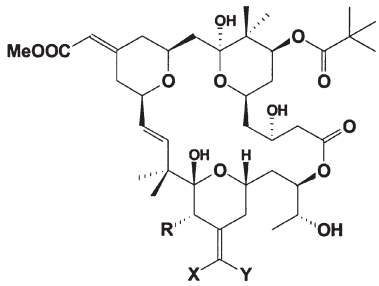
124 Eryloside C,
R = Gal(1,4)Gal(1,3)Gal(1,2)
125 Eryloside D,
R = Gal(1,3)Gal(1,2)



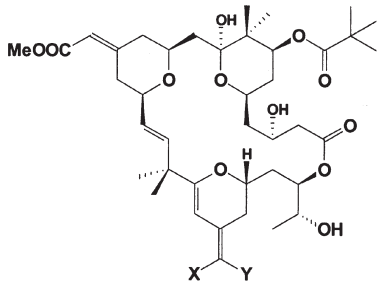
126 Eryloside E
R = Gal(1,2)NAcGal



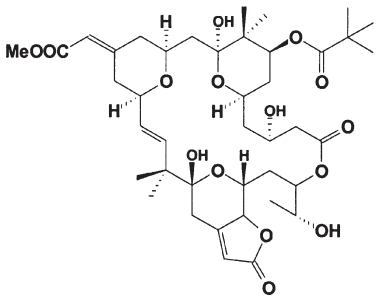
127 Eryloside I, R = CH_2OH
128 Eryloside J, R = H



129 Bryostatin 10, R = H, X = H, Y = COOMe
130 Bryostatin 14, R = OH, X = COOMe, Y = H
131 Bryostatin 18, R = H, X = COOMe, Y = H



132a Bryostatin 16, X = H, Y = COOMe
132b Bryostatin 17, X = COOMe, Y = H



133 Bryostatin 20

ictyalis, Aplidium californium, and *Amathia convoluta* (168). Each organism was discovered to have *B. neritina* growing within its biomass.

Twenty bryostatins have so far been isolated from these two organisms, which are indigenous to the Gulf of Mexico (Florida), Gulf of Aomori (Japan), and Radio Island Jetty near Morehead, North Carolina (USA) (168–170). All bryostatins possess a 20-membered macrolactone in which there are three remotely functionalized pyran rings interconnected by an (*E*)-disubstituted alkene and a methylene bridge. In several bryostatins such as 10, 14, 16, 17, 18, and 20 (**129–131,132a,b,** and **133**), pivalic acid is present.

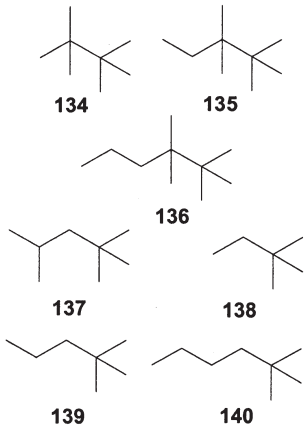
OCCURRENCE OF NEO ALKANES AND DERIVATIVES IN NATURE

Highly branched alkanes have been found in bacteria, cyanobacteria, plants, invertebrates, and other animals. They also are found in sediments, meteorites, and other geological samples (16,17).

Analysis of volatile components of the marine red alga *Corallina elongata* led to the identification of 141 components including three neo alkanes, 2,2,3,3-tetramethylbutane (**134**), 2,2,3,3-tetramethylpentane (**135**), and 2,2,3,3-tetramethylhexane (**136**) (12). Neo alkanes **137**–**140** were produced by *Pseudomonas oleovorans* GPo1 and *Corynebacterium urealyticum* CIP-I-2126 (14,15).

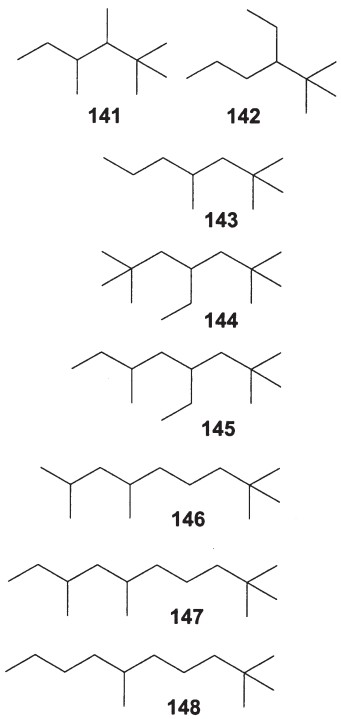
Analysis of the cultured soil cyanobacterium *Microcoleus vaginatus* from the Negev Desert found that this microorganism produced an unusual mixture of four normal and more than 60 branched alkanes, as well as a number of FA, cyclic and unsaturated hydrocarbons, aldehydes, alcohols, and ketones, including some neo alkanes (**141–148**) (13).

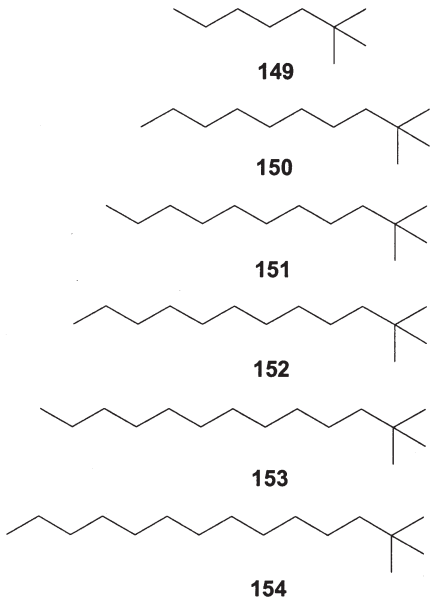
Some neo alkanes (**137**,**149–160**) have been isolated from plants. In cooked samples of black-salsify (*Scorzonera hispanica*), a member of the Asteraceae family, were found 114 com-



ponents, including two neo alkanes (**137** and **155**) (171). From the volatile oils of *Houttuynia cordata* 'Chameleon' was identified 2,2-dimethylheptane (**149**) (172). The volatiles from fresh flowers and leaves of *Gentiana lutea*, *G. punctata* (yellow *Gentiana* spp.), and *G. asclepiadea* (Gentianaceae Juss.) were analyzed by GC/MS and 81 compounds identified, including 2,2-dimethyldecane (**150**) (173,174); compound **150** was also found in dehulled sesame seeds and in volatile compounds of black tea (175).

2,2-Dimethylundecane (**151**) was extracted with a mixture of *n*-pentane, methylene chloride and diethyl ether from samples of Cuban sugarcane molasses (176). Two neo alkanes, 2,2-dimethyldodecane (**152**) and 2,2-dimethyl-ridecane (**153**), were present in alcohol extracts of the fruits of *Averrhoa carambola*; and 2,2-dimethyltetradecane (**158**) was isolated from essential oil of *Plectranthus coleoides* (177,178). The highly branched alkane 2,2,4,6,6-pentamethylheptane (**156**)



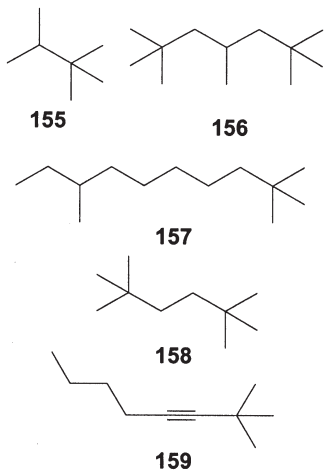


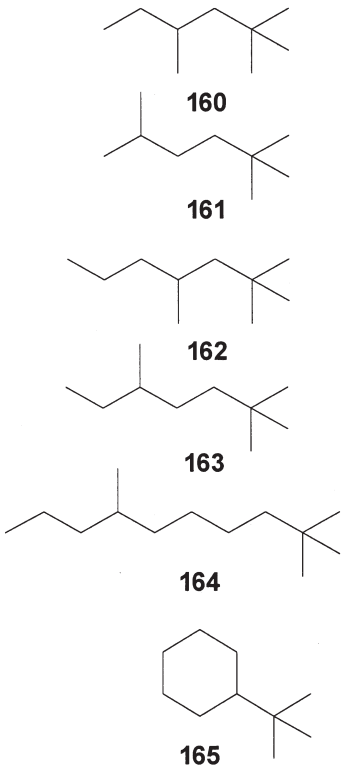
was identified in ginseng root oils; it also was found among volatiles from litter and soil associated with *Ceratiola ericoides*, and in the essential oil of *Anthemis montana* (Asteraceae) (179–181). Two other neo alkanes, 2,2,5,5-tetramethylhexane (**158**) and 2,2,8-trimethyldecane (**157**), were isolated from the volatile components of fresh, tree-ripened apricots (*Prunus armeniaca*), plums (*Prunus salicina*), and their interspecific hybrids (182).

2,2,4,6,6-Pentamethylheptane (**156**) was isolated from the mycelium of the fungus *Tuber borchii* (Ascomycete) grown in a sterile liquid medium (183).

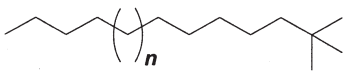
The rare acetylenic neo alkane 2,2-dimethyl-3-octyne (**159**) was detected in essential oil of *Paulownia tomentosa* flowers (83). The neo alkanes **156** and **160** were detected in fresh-cut fruits of pineapple (*Ananas* sp.) (184).

Some neo alkanes and other volatile compounds have been isolated from food products. 2,2,4,6,6-Pentamethylheptane (**156**) and four alkanes (C_{15}–C_{17}, C_{19}) were present among volatile components from freshly picked out meat of blue crab

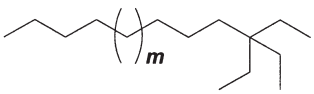




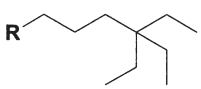
(*Callinectes sapidus*) and processing by-product, and in freshly boiled crayfish tail meat and hepatopancreas (185,186). Alkanes **137** and **156** were present in cooked beef from the local Spanish cattle breeds, Asturiana, Avilena, Parda Alpina, Pirenaica, Retinta, Rubia Gallega, and Morucha, and in raw beef from Asturiana de los Valles, Morucha, Parda Alpina, Pirenaica, and Retinta cattle breeds (187,188). 2,2,5-Trimethylhexane (**161**) was detected in flavor and aroma volatiles of Colonnata bacon, a traditional delicacy (189,190).



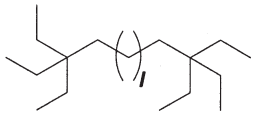
166-175 *n* = 1,2,4,6,8,10,12,14,16



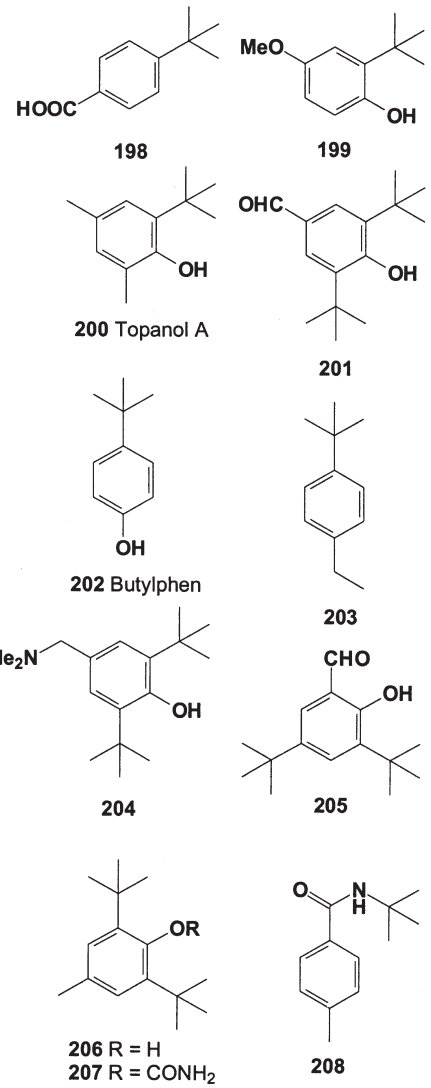
176-183 *m* = 1,2,4,6,8,12,14,16



184-191 R = C_{27}-C_{39}



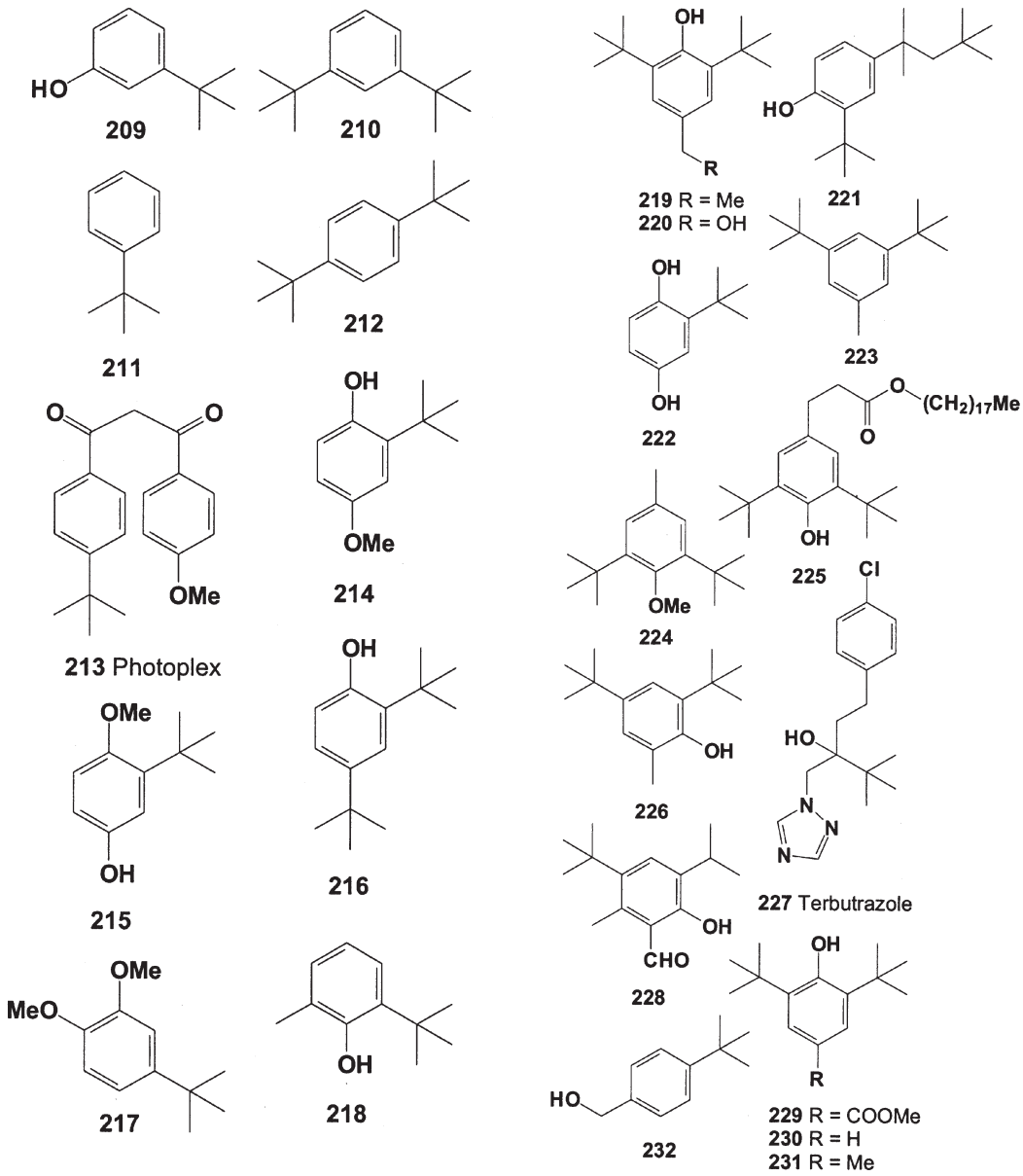
192-197 *l* = 1,4,6,8,10,12



Among 81 compounds, alkane **156** was detected in the volatile flavors of six types of cheese: Parmigiano Reggiano, Fontina, Mahon, Comte, Beaufort, and Appenzeller, and compounds **150** and **151** were isolated from Cheddar and Swiss cheeses during ripening for 9 weeks at 11 and 21°C, respectively (191,192). The aroma fraction of Parmesan cheese was studied in 21 samples of certified origin and aging. Volatile compounds were isolated by means of GC and GC/MS. One hundred sixty-seven compounds were identified: 23 hydrocarbons, including compound **150**, 19 aldehydes, 19 ketones, 29 alcohols, 24 esters, and 25 acids, and 28 other compounds that represented other classes of substances (192,193).

The volatile compounds generated in meat from Iberian and lean pigs after four different treatments (raw, refrigerated, cooked, and refrigerated cooked meat) were analyzed. The different treatments showed different volatile profiles, and all fractions contained the neo alkane **156** (194).

Chicken fat was cooked to 80°C and extracted using supercritical carbon dioxide at 40°C and each of three pressures: 10.3, 20.7, and 31.0 MPa. Of the 318 volatile compounds detected, 99 were classified, including neo alkanes **161**–**164** (195,196).



Raw and smoked black bream (*Brama raii*) and rainbow trout (*Oncorhynchus mykiss*) contained neo alkane **156** (74). The same compound was detected in volatile components of eight cultivars of potato after microwave baking (197). Coryneform bacteria produced *tert*-butylcyclohexane (**165**) (198).

Branched alkanes, such as dimethylalkanes and monomethylalkanes, are common in organic geological samples. At present, the sources of these identified compounds are not well known. Some short-chain (C_{16}–C_{21}) dimethylalkanes (**166–168**) and monomethylalkanes were identified in cyanobacterial cultures, fungi, and marine algae (12–15,199). 2,2-Dimethyldocosane was tentatively identified in the desulfurized polar fraction of an Oligocene anhydrite (200), and a pseudohomologous family of 2,2-dimethylalkanes (C_{16}–C_{28}) (**166–175**) was tentatively identified in Cenomanian and Turonian black shales of Canada (201). Unusual pseudohomologous series of branched alkanes with one (**176–183**) or two quaternary (**192–197**) carbon atoms were present in the extractable organic matter of Cenomanian and Turonian black shales of Pasquia Hills (Saskatchewan, Canada) (202). The 3,3-diethylalkanes ranged from C_{15} to C_{39} (**184–191**) with C_{max} at 27, and only odd carbon-numbered pseudohomologs were detected in the sulfide deposits of the Rainbow field (Mid-Atlantic Ridge) (203). The origin of these compounds remains unclear, although a bacterial origin is favored.

OTHER NATURAL METABOLITES CONTAINING A TERTIARY BUTYL GROUP(S)

Some aromatic compounds containing *tert*-butyl unit(s) can be components of natural antioxidants, and they have been isolated from plants, marine algae, and fungi (204,205).

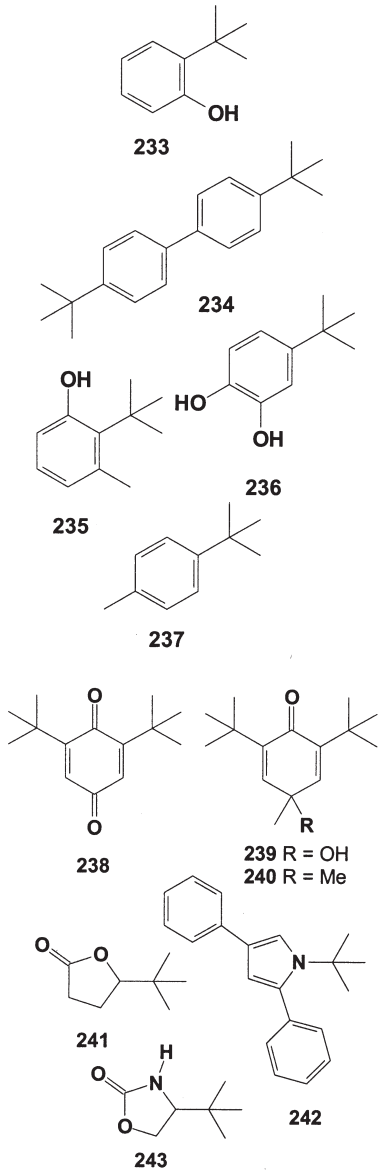
4-*tert*-Butylbenzoic acid (**198**) and 4-methoxy-2-*tert*-butylphenol (**199**) were found among volatile substances in

TABLE 1
Distribution of Aromatic and Other Metabolites with a Tertiary Butyl Group(s)

Compounds	Sources	Part	Ref.	Compounds	Sources	Part	Ref.
206,**210**,**212**	*Acanthopanax gracilistylus*	Root, bark	215	**226**	*Sparassis crispa*	Roots	253
206,**210**	*Acanthopanax gracilistylus*	Bark	216	**231**	*Stellaria dichotoma*	Roots	254
228	*Afrostyrax kamerunensis*	Bark	217	**220**	*Symplocos sumunlia*	Leaves	255
228	*Afrostyrax lepidophylleus*	Bark	217	**219**,**220**,**223**	Xinkaihe ginseng	Roots	236
214	Aloe *arborescens*	Leaves	218	**211**	*Zanthoxylum bungeanum*	Leaves	256
202	*Angelica* sp.	Roots	219				
210,**209**	Apple tree (*Malus domestica*)	Apple-bearing twigs	220	**217**	*Zeravschania pastinacifolia*	Leaves	237
221	*Artemisia scoparia*	Whole	221	**232**	*Pinus densiflora*	Needles, sprouts, twigs	257
234	*Aspergillus fumigatus* F93	Fermentation broth	222	**247**,**248**	*Ambrosia artemisifolia*	Roots	258
				248	*Ambrosia trifida*	Roots	258
231	Bamboo tree (*Phyllostachys pubescens*)	Shoots	223	**248**	*Ambrosia trifoliata*	Roots	258
231	Blackberry (*Rubus* sp.)	Whole	224	**239**	*Arillus longan*	Whole	259
213	Black currant (*Ribes nigrum*)	Fruits	225	**238**	Black tea and green tea	Leaves	226
206	Black and green tea	Leaves	226				
215,**216**	Black truffle (*Tuber melanosporum*)	Whole	227	**241**	*Citrus junos*	Fruits	232
				243	Crayfish	Waste	260
230	Brown marine alga (*Undaria pinnatifida*)	Whole	228	**245**	*Dalbergia odorifera*	Leaves	261
		Whole	228	**249**	Garden	Waste	262
222	*Chrysanthellum indicum*	Leaves	229	**247**	*Iva xanthifolia*	Roots	258
206,**214**,**229**	*Chrysosplenium nudicaule*	Leaves	230	**238**	*Lamium maculatum*	Leaves	263
231	*Codonopsis pilosula*	Roots	231	**241**	Mango (*Mangifera indica*)	Fruits	241
211,**219**	*Citrus junos*	Fruits	232				
231	*Elsholtzia patrini*	Roots	233	**247**,**248**	*Melampodium divaricatum*	Roots	258
231,**233**	*Filipendula palmata*	Leaves	234				
224	Green tea	Leaves	235	**247**	*Melampodium longifolium*	Roots	258
228	*Hua gabonii*	Bark	217				
219,**220**,**223**	Japanese ginseng	Roots	236	**238**	*Nepeta mussini*	Roots	263
219,**220**,**223**	Korean white ginseng	Roots	236	**231**,**246**	*Panax ginseng*	Roots, leaves	264
217	*Leutea glaucopruinosa*	Leaves	237	**240**	*Rabdosia macrocalyx*	Aerial	250
218	*Lippia graveolens*	Leaves	238	**247**,**248**	*Rudbeckia hirta*	Roots	258
201	*Lysimachia foenum-graecum*	Leaves	239	**247**,**248**	*Rudbeckia speciosa*	Roots	258
				243	*Salix matsudana*	Leaves	265
213	*Malpighia emarginata*	Seeds	240	**247**,**248**	*Schkuhria advena*	Roots	258
219	Mango (*Mangifera indica*)	Fruits	241	**247**,**248**	*Schkuhria pinnata*	Roots	258
198	*Melaleuca* cajuputi	Leaves	242	**247**,**248**	*Schkuhria senecioides*	Roots	258
206	*Nelumbo nucifera*	Leaves	243	**242**	*Strychnos cathayensis*	Whole	266
237	*Nepeta leucophylla*	Roots	244	**239**	*Symplocos sumunlia*	Leaves	255
201,**225**–**227**	Oak wood (*Quercus*)	Sawdust	245	**250**	*Aspergilus flavus*	Whole	267
235	*Ostericum grosseserratum*	Roots	246				
206	*Panax ginseng*	Leaves	247				
206,**207**	*Panax ginseng*	Aerial	248				
216	*Psoralea corylifolia*	Roots	249				
201	*Rabdosia macrocalyx*	Aerial	250				
206	Red tea (Qimen)	Leaves	251				
228	*Scorodophloeus zenkere*	Bark	217				
235	*Siphonostegia chinensis*	Roots	252				

fruits of *Zizyphus jujube* (206). The well-known antioxidant topanol A (**200**) was found in dry and fresh flowers of *Aloe vera* (207), and identified from the green alga *Enteromorpha compressa* and the red alga *Hypnea musciformis* (208). The 4-formyl-2,6-di-*tert*-butylphenol (**201**) was among 62 compounds detected in odors of apple trees (209). Butylphen (**202**) and compound **204** were detected in the volatiles of fresh flowers from nine natural populations of four *Lamium* species: *L. album, L. galeobdolon, L. garganicum, L. maculatum*, and *L. purpureum* (210). Thirty-three components have been identified in the odors emitted by fresh flowers of *Fissistigma shangtzeense*, which is found in Guangxi and the southwest of Yunnan province of China, including 3,5-di-*tert*-butylsalicylic aldehyde (**205**) (211). Essential oil from flowers of *Robinia pseudoacacia* contained approximately 51% α-limonene, 12% γ-terpinene, and 7% 2,6-di-*tert*-butyl-4-methylphenol (**206**), and minor compound **207** (212). Floral fragrance oils related to pollination were studied in three cacao species of *Theobroma* and one *Herrania* species (Sterculiaceae) in Costa Rica, and N-*tert*-butyl-4-methylbenzamide (**208**) was found (213).

Two fungal species, *Penicillium commune* and *Paecilomyces variotii*, were cultivated on pine wood and on a combination of

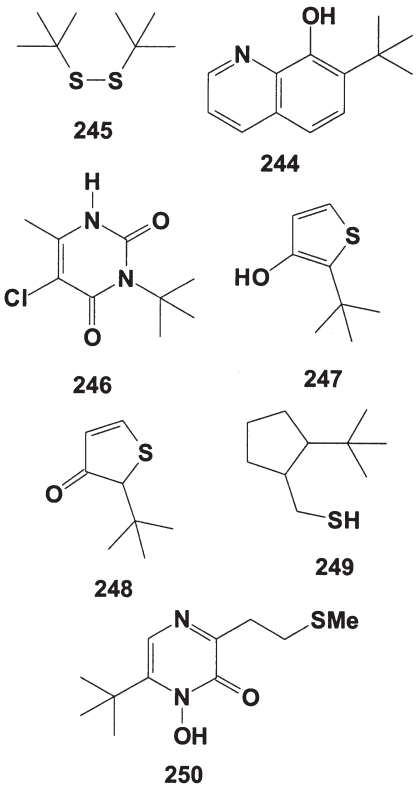


gypsum board and mineral wool, and both fungal species produced *p-tert*-butyl-ethylbenzene (**203**) (214).

About 40 natural aromatic compounds containing *tert*-butyl unit(s) (**198**–**237**) have been isolated from plant, marine algae, and fungi, their distribution in nature shown in Table 1. Occurrence other metabolites with *tert*-butyl unit(s) (**238**-**250**) shown in Table 1.

Some novel natural metabolites with a tertiary butyl group(s) have been discovered in plant species and fungi. Two new sulfur-containing derivatives, *tert*-Bu-3-[(1-methylpropyl)dithio]-2-propenyl malonate (**251**) and *tert*-Bu-3-[(1-methylthiopropyl)thio]-2-propenyl malonate (**252**) were isolated from the roots of *Ferula persica* var. *latisecta* grown in Iran (268).

The roots of *Toona ciliata* (Brazil) yielded a new 9,10-dihydrophenanthrene, which was identified as 9,10-dihydro-9-hydroxy-9-(*tert*-butoxycarbonylmethyl)-10-oxo-phenanthrene (**253**) (269).



Among four furanocoumarins isolated from the roots of *Angelica dahurica* (grown in South Korea) was a new bioactive oxypeucedanin hydrate-3″-*tert*-Bu ether (**254**) (270).

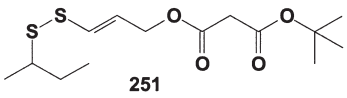
The essential oil steam distillation of the aerial parts of *Saussurea arenaria*, *S. iodostegia* and *S. japonica* led to a 4-*tert*-butoxyanisole (**255**) (271). A derivative of 1(2H)-pentalenone (**256**) was extracted from and detected in aged flue-cured tobacco leaves (272).

Two stereoisomers (**257** and **258**) were produced by the filamentous fungus *Penicillium citreo-viride* (273). S-*tert*-Butyl-L-cysteine (**259**) was obtained by the cleavage of alliin by C-S-lyase isolated from ramson, the wild garlic (*Allium ursinum*) (274). Compound **260** was obtained by biotransforming *cis*-2-methyl-3-oxobutanoic acid ethyl ester with bakers' yeast at 30°C for 48 h (275). The volatile amines **261** and **262** were detected in dry bakery yeast autolyzate (276). Three cyclohexanol derivatives (**263**–**265**) and compound (**266**) were detected in essential oil of medicinal herb extract (277).

BIOLOGICAL ACTIVITIES OF NEO ACIDS AND DERIVATIVES

Many natural metabolites and synthesized compounds (**267**–**289**) containing a tertiary butyl group(s) show biological activity, and some of them used in clinical medicine. Some synthesized compounds with *tert*-butyl unit(s) for which activity has been demonstrated activities are considered next.

Photoplex (**213**) has other names: escalol 517, eusolex 9020, neoheliopan 357, parsol 1789, parsol A, parsol RTM 1789, and uvinul BMBM. It has a broad spectrum of protective proper-



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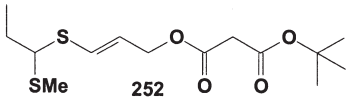

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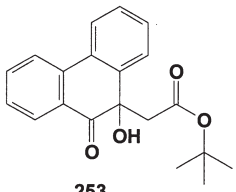

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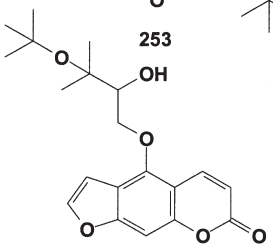

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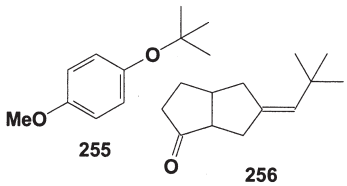

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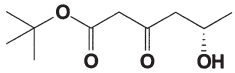

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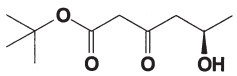

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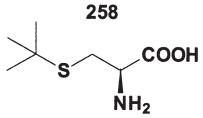

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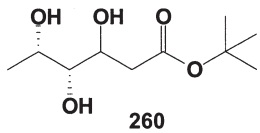

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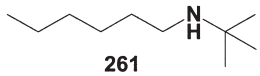

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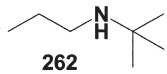

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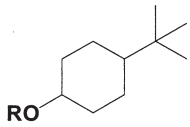
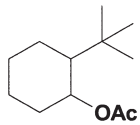


263 R = H
264 R = Ac



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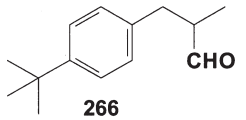

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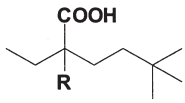
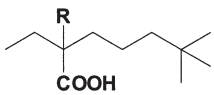
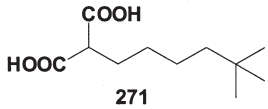


267 R = H
268 R = COOH



269 R = H
270 R = COOH



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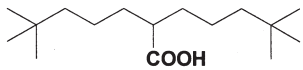

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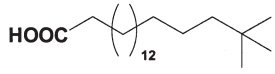

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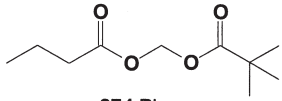
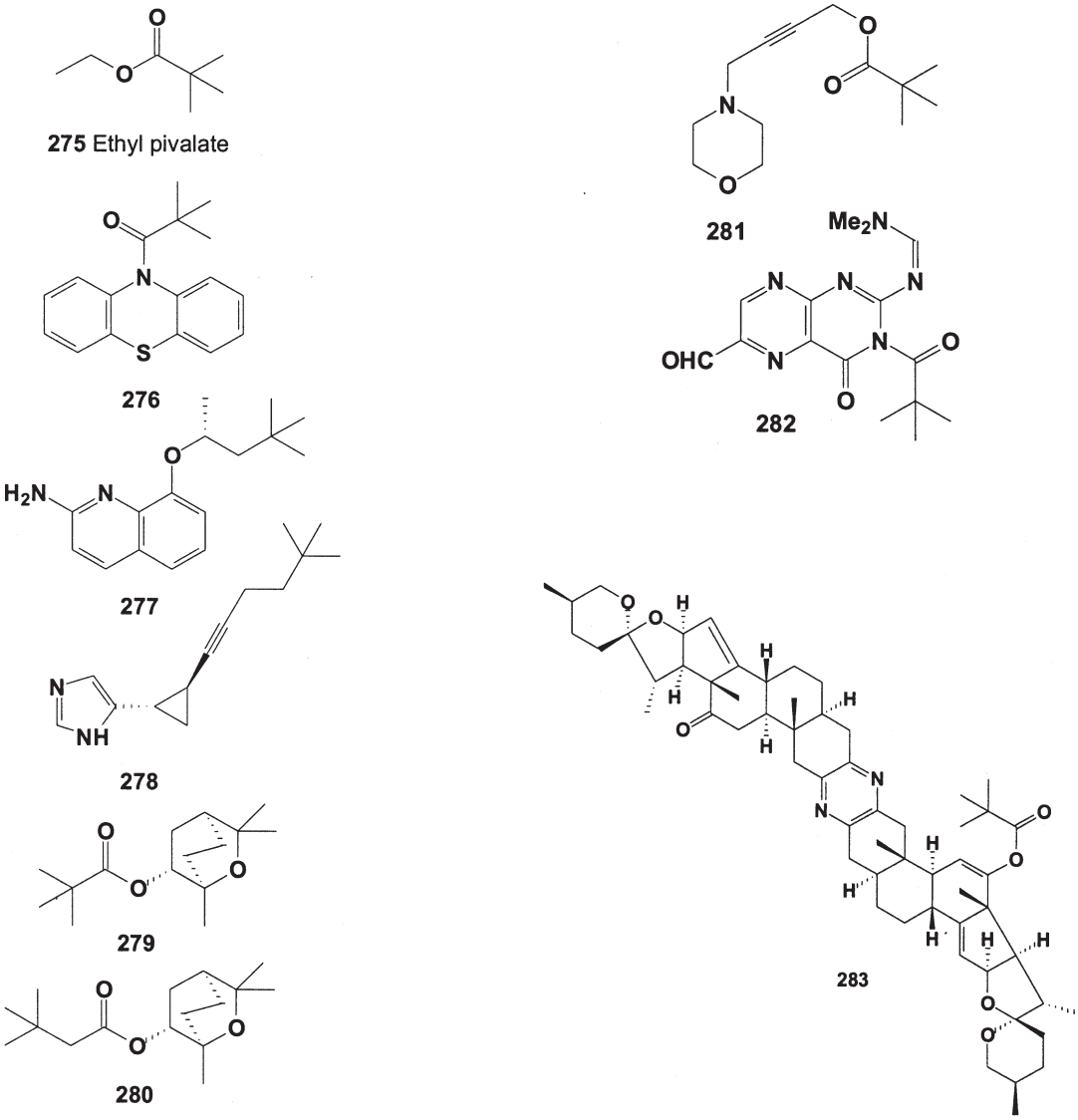


274 Pivanex

ties toward human skin, and it is used in clinic dermatology (278,279).

A novel biphenolic compound, bis(2-hydroxy-3-*tert*-butyl-5-methylphenyl)-methane (GERI-BP002-A, **234**), that was isolated from the fermentation broth of *Aspergillus fumigatus* F93 (222) is cytotoxic to human solid tumor cells. Effective doses that cause 50% inhibition of cell growth *in vitro* against non-small cell lung cancer cell A549, ovarian cancer cell SK-OV-3, skin cancer cell SK-MEL-2, and central nerve system cancer cell XF498 are 8.24, 10.60, 8.83, 9.85 μg/mL, respectively.

Neo FA (**14** and **267**–**272**) were prepared and showed strong bactericidal properties (280). 12,12-Dimethyltridecanoic acid (**21**) and 18,18-dimethylnonadecanoic acid (**273**) exhibited synergistic effects in combination with tetramethylthiuram-disulfide, and strongly enhanced the effect of conventional antimicrobial agents that act inside the cell membrane (281). A novel derivative of butyric acid, pivalyloxymethyl butyrate (pivanex, or AN-9, **274**) has been shown, *in vitro*, (i) to induce cy-



todifferentiation and inhibit the proliferation of leukemic cells; and (ii) to inhibit the growth and formation of Lewis lung carcinoma colonies in semisolid agar. AN-9 is a potential anti-neoplastic agent (282) that induces cell death by apoptosis (283).

Ethyl pivalate (**275**) has demonstrated antitumor activity against Ehrlich L-1210 leukemia (284). Compound pivaloylphenothiazine, **276**, showed 24.3% inhibition of Ehrlich-type tumors in male mice (285). 2-Amino-8-alkoxy quinoline (**277**) was synthesized and had properties as a melanin-concentrating hormone receptor-1 (MCHr1) antagonist (286).

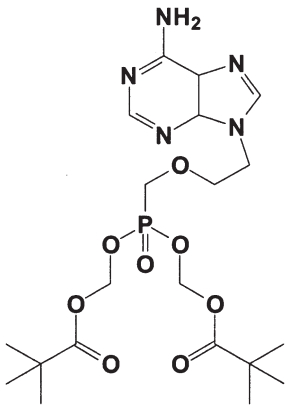
Dimethylpentynylcyclopropylimidazole (**278**) (GT-2331 or Cipralisant), a potent histamine H3 antagonist, was prepared, and biological activity was also reported (287,288). The alkyl esters of 2-endo-hydroxy-1,8-cineole (**279** and **280**) were synthesized, with yields of 57.8-98.0%. The antimicrobial and bactericidal activities of these synthetic esters against bacteria (*Staphylococcus aureus, Escherichia coli*, and *Pseudomonas fluorescens*) were reported (289). As a result, the *tert*-Bu acetate of compound **280** showed the highest antimicrobial and bactericidal activities against all kinds of the test bacteria (289).

The 4-morpholino-2-butynyl pivalate (**281**) was synthesized and of the eight examples it had the highest antitumor activity and low toxicity to fibroblasts (290).

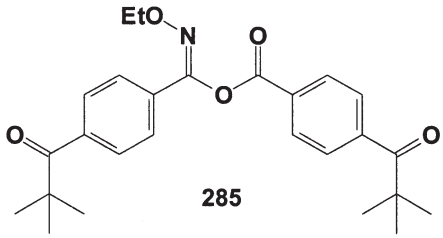
A novel variant of 6-formylpterin (6FP), 2-(N,N-dimethylamino-methylene-amino)-6-formyl-3-pivaloyl-pteridine-4-one (6FP-tBu-DMF, **282**) was synthesized, and photodynamic effects on a pancreatic cancer cell line (Panc-1 cells) were observed (291). A cephalostatin analog (**283**) was highly effective in inhibiting cell growth in various human cell lines (292).

Antiviral agent **284** showed strong activity against human hepatitis B virus and herpes viruses 1, 2, and 5 (293). Pivaloylbenzoyl N-alkoxypivaloylbenzimidates (**285** and **286**) were prepared and used as antidiabetic and anticholesterolemic agents (294).

Following oral administration in mice, organ levels of pivampicillin (**287**) were three- to fourfold greater than the levels of ampicillin. The *in vitro* antibacterial activity of compound **287** against a number of Gram-negative and Gram-positive bacteria was similar to that of ampicillin. Also compound **287** was less sensitive than ampicillin to inactivation by streptococcal β-lactamase (295).



284 Hepsera



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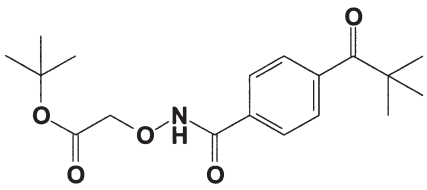

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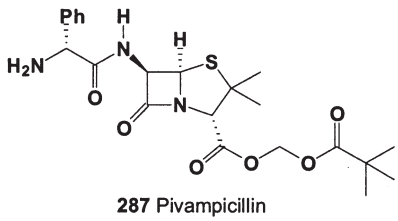
Pivmecillinam (**288**) is a pivaloyloxymethyl ester of a new penicillin analog that is hydrolyzed after absorption by nonspecific esterases to release mecillinam. Comparable studies suggested that standard doses of pivmecillinam were unlikely to be effective in the majority of cases of urinary infection by fecal streptococci (Micrococcaceae) (296). The *O*-pivaloyltyrosine derivative (**289**) was prepared, and it showed elastase-inhibiting activity at IC_{50} of 0.84 μM (297).

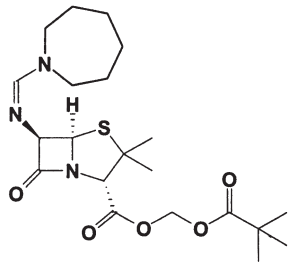
APPLICATION OF NEO ACIDS AND DERIVATIVES

Different pivalic acid derivatives and/or compounds with a tertiary butyl group(s) are produced and used in the cosmetic, agronomic, and perfume industries. Skin creams and lotions contain a pivaloylascorbic acid (**290**) and pivaloyl derivates (**291**-**293**) as well as derivatives of vitamin E, including vitamin E acetate, vitamin E nicotinate, and vitamin E orotate. These cosmetics have little irritating effect on skin, and they condition the skin for a long period of time (298,299). Two seco-theaspiranes (**294**,**295**) with pronounced blackcurrant notes were synthesized as odor and odorous substances and used in cosmetics (300).

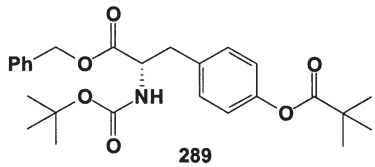
Derivatives of neo FA (**296**–**300**) are used in the cosmetic industry, for example, by L'Oreal (France) (301). An amide of a trialkylacetic acid (**301**), amides of neo FA (**302**,**303**) and a



287 Pivampicillin



288 Pivmecillinam



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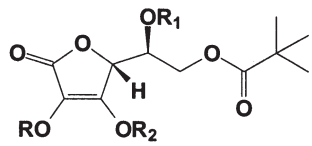
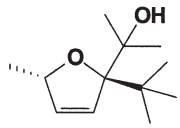


290 R = R_1 = R_2 = H
291 R = Pivaloyl, R_1,R_2 = H
292 R,R_1 = Pivaloyl, R_2 = H
293 R,R_1,R_2 = Pivaloyl



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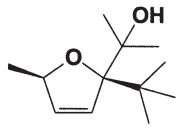
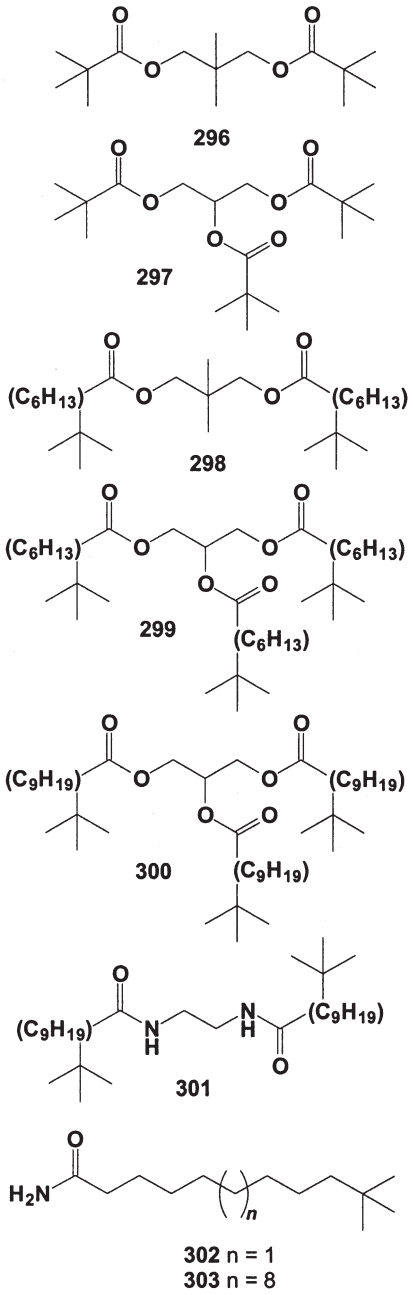
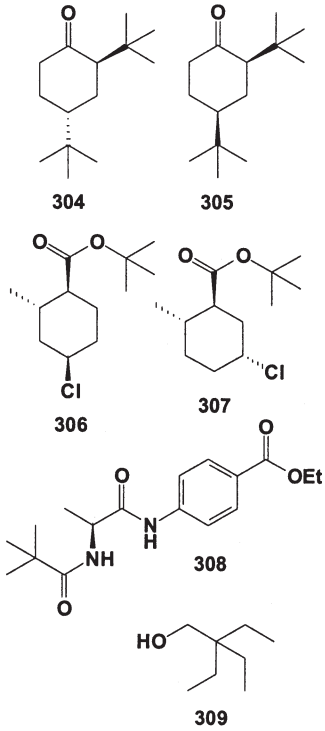


295

polyamine were used with a particulate carrier (e.g., detergent builder or filler) or liquid medium (e.g., aqueous nonionic surfactant solution) in a composition that was added to fabrics in a laundry bath to impart antistatic properties to fabrics after laundering and automatic machine drying (302,303).

2,4-Di-*tert*-butylcyclohexanones **304** and **305** have been synthesized and used as components in perfume. Compound **304**) has a mild, soft, floral-woody and violet-like scent. An equal mixture of *cis*- and *trans*-isomers of compound **04** has a mild, soft, floral-woody and violet-like scent. The *cis*-isomer





of **304** by has a highly diffusible cyclamen-like and floral scent; the *trans*-isomer of compound **304** has an orris-like woody scent (304).

The most biologically active forms of the insect attractant trimedlure, a synthetic lure used to attract male Mediterranean fruit flies, are the *tert*-Bu esters of *cis*-4- (**306**) and *trans*-5-chloro-*trans*-2-methyl-cyclohexanecarboxylic (**307**) acids (305).

The peptide analog of insect juvenile hormone ethyl pivaloyl-D-alanyl-*p*-aminobenzoate (**308**) had pronounced systemic activity in sunflowers for controlling *Dysdercus cingulatus* (306). This compound can enter the plant through the leaves or the roots, and is translocated throughout the plant in an active form. There was nearly maximal activity at 500–1000 μg (**308**)/plant, and activity remained for up to 14 d.

2,2-Diethylbutyric acid (**49**) forms stable dispersions and is used to achieve long-lasting fragrance or aroma at room temperature in a paint-scenting additive mixture. This mixture has good coverage ability, and its fresh fragrance lasts for over 180 d (307). 2,2-Diethyl-1-butanol (**309**) has been used as a minor ingredient in aquatic ink (308).

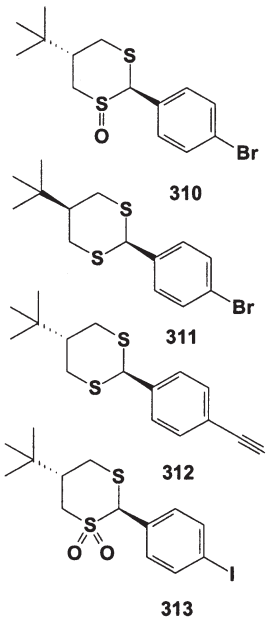
The 1,3-dithianes and 1,3-dithiane 1,1-dioxides (**310**–**313**) are more effective insecticidal compounds in the dithiane series than known halogenated compounds (hexachlorocyclohexanes, polycholorobornanes, and chlorinated cyclodienes) against the housefly (LD_{50} from 0.36 to 0.0095 μg/g) (309).

CONCLUSION

Neo fatty (carboxylic) acids as well as other metabolites containing a tertiary butyl group(s) belong to a rare group of natural products. In the last 100 years fewer than 300 representatives from this rare and unique group of secondary metabolites have been identified. However, many different natural and synthesized compounds with *tert*-butyl group(s) show high biological activities. Simple aromatic compounds with *tert*-butyl group(s) demonstrated good antioxidant and fungicidal properties. The presence of *tert*-butyl amino acids that are incorporated into structures of natural peptides and/or natural alkaloids found in cyanobacteria, and marine invertebrates, or free *tert*-butyl amino acids found in some fungi, and microorganisms are worthy of note.

Neo acids and derivatives have a low order of toxicity and are used by different chemical companies for synthesis of novel biologically active compounds in the fields of agronomy, medicine, and cosmetics.

Novel natural neo FA and their analogs and derivatives have



been discovered and evaluated for their biological activity. It seems certain that some possess anticarcinogenic, antifungal, antiviral, and/or antibacterial properties. Certainly other important neo FA derivatives, and/or metabolites with *tert*-butyl unit(s) possessing biological activity also will be discovered and/or synthesized.

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Gestational Diabetes Mellitus Enhances Arachidonic and Docosahexaenoic Acids in Placental Phospholipids

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ABSTRACT: In previous studies, we reported that neonates of women with gestational diabetes mellitus (GDM) have reduced blood levels of arachidonic acid (AA) and docosahexaenoic acid (DHA) that were unrelated to maternal status. Since both AA and DHA are selectively transferred from maternal to fetal circulation by the placenta, we have investigated whether the FA composition of the placenta is altered by GDM. Thirty-six women, 11 with and 25 without GDM, were recruited from Newham General Hospital, London. The women with GDM had higher levels of di-homo-γ-linolenic ($P < 0.05$), docosatetraenoic (n-6 DTA; $P < 0.0001$), docosapentaenoic n-6 ($P < 0.005$), total n-6 ($P < 0.005$), docosapentaenoic (n-3 DPA; $P < 0.005$), and total n-3 ($P < 0.01$) FA, as well as higher levels of AA ($P < 0.05$) and DHA ($P < 0.01$), in placental choline phosphoglycerides (CPG) compared with the healthy women who served as controls. Similarly, the women with GDM had elevated n-6 DTA ($P < 0.005$), AA, total n-6 metabolites ($P < 0.05$), DHA, total n-3 metabolites, and total n-3 FA ($P < 0.005$) in ethanolamine phosphoglycerides (EPG). In contrast to CPG and EPG, the placental TG of the women with GDM had higher linoleic acid ($P < 0.05$) and lower AA, n-6 metabolites, and n-3 DPA ($P < 0.01$). The placenta is devoid of desaturase activity, and it is thought to be reliant on maternal circulation for both AA and DHA. Hence, the enhanced levels of the two FA in the placenta of the GDM group suggests that these FA are taken up from the maternal circulation and retained after esterification into phosphoglycerides instead of being transferred to the fetus. Further study is needed to elucidate the mechanism involved and the effect of the phenomenon on postnatal growth and development of the offspring.

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Both first- and second-phase insulin secretion increase in the later part of pregnancy (1) to compensate for pregnancy-induced insulin resistance and to maintain normal carbohydrate tolerance. Some women, 3–5% (2,3), fail to respond to the changes in insulin resistance and develop a transient diabetes, gestational diabetes mellitus (GDM), that resolves after delivery.

In human (types 1 and 2) and experimental diabetes, the activity of Δ-6 and Δ-5 desaturase, enzymes that are vital for the synthesis of arachidonic acid (AA) and DHA, is impaired (4–6), and the levels of both desaturases are reduced in membranes (7–9). AA and DHA are vital for the structure and function of cell and subcellular membranes (10), and the requirement for these FA increases during pregnancy (11,12).

Although the fetus and neonate are able to synthesize AA from linoleic acid (LA) and DHA from α-linolenic acid (13–17), the rate of synthesis is not fast enough to support optimal growth and development (18–20). Consequently, it is now acknowledged that the fetus is reliant on the mother for an optimal supply of these nutrients. In pregnancy complicated with diabetes, owing to the impairment of AA and DHA synthesis in the mother, there may be an imbalance between the fetal requirement and maternal supply. Indeed, there is evidence that AA and DHA levels are compromised in neonates of women who have type 1 (21), type 2 (22), and gestational (23,24) diabetes. Compared with healthy controls, the women with GDM had lower AA and DHA levels in red cells (25) but not in plasma (26). Because the maternal plasma AA and DHA, which is the main source for the fetus, was normal in the women with GDM, it is unclear whether the observed compromise in the level of the two FA in the neonates (23,24) was due to reduced supply, impaired transfer, or both. The aim of this study was to investigate whether GDM alters the placental FA profile.

MATERIALS AND METHODS

Subjects. Thirty-six women, 11 women with GDM and 25 women who were healthy controls, with uncomplicated singleton pregnancies were recruited on admission for delivery at the Newham General Hospital, London, UK. The women with GDM comprised four of African descent and seven of Asian descent. Of the control women, six were Caucasian, six African, and 13 Asian. The mean pre-pregnancy body mass index (BMI) and age of the control and GDM groups were 25.9 ± 4.4 and 28.6 ± 5.4 (range 20–41) and 30.1 ± 3.5 and 32.6 ± 3.3 (range 27–37), respectively. The women in the control group did not have a family history of diabetes, high

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Abbreviations: AA, arachidonic acid; BMI, body mass index; CPG, choline phosphoglycerides; DHGLA, di-homo-gamma-linolenic acid; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; EPG, ethanolamine phosphoglycerides; GDM, gestational diabetes mellitus; LA, linoleic acid; LCPUFA, long-chain PUFA.

blood pressure, or other chronic disorders. Ethical approval from the East London Health Authority and written consent from the subjects were obtained.

Diabetes diagnosis, treatment, and outcome. As part of the antenatal care, and because of the high prevalence of type 2 diabetes in this borough of East London, all of the women were assessed for the presence of the disease at 16 wk of gestation. In addition, those women considered to be at high risk of GDM were screened with an oral glucose tolerance test at gestation week 28. Women were considered to be at risk if they had a BMI greater than 30, polycystic ovary syndrome, or a history of GDM, insulin resistance, pregnancy-induced hypertension, stillbirth, or macrosomia. Blood sugar levels were determined after an overnight fast. Those with a glucose concentration of greater than 7 mmol/L were given a high-energy carbohydrate supplement, Polycal (Nutricia Zoetermeer, The Netherlands), equivalent to 75 g glucose load by diluting 113 mL of Polycal to 200 mL with water. GDM was diagnosed if the glucose concentration at 120 min was greater than 7.8 mmol/L.

Subsequent to diagnosis, four of the GDM subjects were treated with diet, four with insulin, one with both insulin and diet, and two did not receive any treatment. None of the subjects in either the GDM or healthy control groups had obstetric or clinical complications, and they all delivered at term. The gestational age, birth weight, and head circumference of the babies of the women in the GDM group were 37.7 ± 0.7 wk, 3251.8 ± 674.6 g, and 33.6 ± 1.7 cm, and the corresponding values for the babies of the women in the healthy control group were 39.7 ± 1.1 wk, 3342.8 ± 597.4 g, and 33.7 ± 1.3 cm.

Sample collection. Immediately after delivery, the placentas were cleaned of traces of blood and weighed. A representative sample was taken from the same area of each placenta, washed several times in cold saline to remove all traces of blood, and stored at –70°C until analysis.

Extraction and analysis of total lipids. Total lipids were extracted by the method of Folch *et al.* (27) from placental homogenates in chloroform/methanol (2:1 vol/vol) containing BHT (0.01% wt/vol) under nitrogen. The total lipid extract was dried under a stream of nitrogen, dissolved in a known volume of chloroform/methanol, and transferred carefully to a preweighed trident vial. Subsequently, the total lipid solution in the vial was dried under a stream of nitrogen to a constant weight. The weight of the total lipid extracted from a known weight of placenta was computed by subtracting the weight of the empty vial from the weight of the vial and total lipid.

Analysis of FA. The total lipid was dissolved in chloroform/methanol (2:1 vol/vol) and an aliquot was taken for FA analysis. The different classes of phospholipids were separated by TLC on silica gel plates with the use of the developing solvents chloroform/methanol/methylamine (65:35:15 by vol), containing 0.01% BHT. Similarly, TG were separated from cholesterol esters and FFA by TLC on silica gel plates with the developing solvents petroleum spirit/diethyl ether/formic acid/methanol (85:15:2.5:1 by vol), containing 0.01% BHT. The separated lipid bands were visualized by spraying the developed plate with a methanolic solution of 2,7-dichlorofluorescein (0.01% wt/vol).

FAME were prepared by heating the lipid fractions in 4 mL of 15% acetyl chloride in methanol for 3 h at 70°C, in a sealed vial under nitrogen. FAME were separated by GLC (HRGC MEGA 2 series; Fisons Instruments, Italy) fitted with a capillary column (30 m × 0.32 mm i.d., 0.25-μm film, BP20). Hydrogen was used as a carrier gas, and the injector, oven, and detector temperatures were 235, 250, and 178°C, respectively. FAME were identified by comparison of retention times with authentic standards and calculation of equivalent chain length values. Peak areas were quantified by a computer chromatography data system (EZChrom Chromatography Data System; Scientific Software Inc., San Ramon, CA).

Statistical analysis. The data are expressed as median and interquartile ranges. The Mann-Whitney U nonparametric test was used to investigate significant differences in demographic and fatty acid data between the two groups of subjects, and between the diabetic and nondiabetic Asian women. Kruskal–Wallis nonparametric ANOVA and the Mann–Whitney U test were used to investigate a statistical difference in AA and DHA between Caucasian, African, and Asian subjects in the control group. $P < 0.05$ was considered to be significant. All of the analyses were performed with the use of the statistical software SPSS for Windows, version 11.5.

RESULTS

Placental weight and total lipids. There was no difference in placental weight between the GDM (640 ± 176.7 g) and the healthy control (573 ± 148.6 g) groups ($P > 0.05$). However, the placental total lipids were elevated in the GDM (15.8 ± 1.0 mg/g) group compared with the control group (14.2 ± 2.3 mg/g) ($P < 0.005$).

Placental FA composition. The median percentage FA of placental choline phosphoglycerides (CPG) and ethanolamine phosphoglycerides (EPG) and TG of the women with and without gestational diabetes mellitus is presented in Tables 1, 2, and 3, respectively.

CPG. The women with GDM had higher levels of stearic ($P < 0.0001$), di-homo-γ-linolenic acid (DHGLA; $P < 0.05$), AA ($P < 0.05$), docosatetraenoic acid (n-6 DTA; $P < 0.0001$), docosapentaenoic acid (n-6 DPA; $P < 0.005$), n-6 metabolites ($P < 0.005$), total n-6 FA ($P < 0.005$), docosapentaenoic acid (n-3 DPA; $P < 0.005$), DHA ($P < 0.01$), n-3 metabolites ($P < 0.01$), and total n-3 FA ($P < 0.01$) compared with the controls. In contrast, the proportions of palmitic, total saturated, and palmitoleic FA were lower in the GDM group ($P < 0.0001$).

EPG. Similar to the CPG, the EPG of the GDM group had elevated proportions of n-6 DTA ($P < 0.005$), AA, n-6 metabolites ($P < 0.05$), DHA, total n-3 metabolites, and total n-3 FA ($P < 0.005$) compared with the control group. However, the GDM group had reduced palmitic ($P < 0.01$), palmitoleic, oleic, total monounsaturated FA, LA ($P < 0.0001$), and DHGLA ($P < 0.05$).

TABLE 1
FA of Placental Choline Phosphoglycerides[a]

	Control (n = 25)		GDM (n = 11)	
	Median	25–75% IQR	Median[b]	25–75% IQR
16:0	38.6	37.3–40.0	32.5****	30.7–34.2
18:0	7.75	7.21–8.27	9.08****	8.94–9.57
ΣSFA	46.4	44.9–47.8	41.5****	39.6–43.1
16:1	1.29	1.16–1.45	1.02****	0.88–1.06
18:1	10.1	9.09–10.9	10.8	10.1–11.4
ΣMono	11.4	10.3–12.4	12.0	11.1–12.8
18:2n-6	12.8	12.1–15.1	14.6	13.9–14.7
20:3n-6	3.78	3.43–4.24	4.65*	4.34–4.87
20:4n-6	17.8	15.8–18.9	19.1*	18.3–20.3
22:4n-6	0.30	0.26–0.36	0.63****	0.40–0.69
22:5n-6	0.22	0.18–0.24	0.36***	0.24–0.49
Σn-6 met	23.2	20.5–24.6	25.7***	24.2–27.3
Σn-6	36.4	33.5–38.0	39.6***	38.4–41.3
18:3n-3	0.07	0.06–0.09	0.07	0.04–0.08
20:5n-3	0.17	0.11–0.24	0.22	0.16–0.28
22:5n-3	0.19	0.16–0.23	0.30***	0.23–0.37
22:6n-3	1.52	1.02–1.90	1.94**	1.79–2.43
Σn-3 met	1.75	1.19–2.14	2.43**	2.08–2.88
Σn-3	1.79	1.26–2.22	2.43**	2.16–2.96

[a]IQR = interquartile range; ΣSFA = total saturates; ΣMono = total monounsaturates; Σn-6 met = total n-6 metabolites = Σ(18:3n-6, 20:2n-6, 20:3n-6, AA, DTA, DPA n-6); Σn-6 = total n-6 FA = Σ(LA, 18:3n-6, 20:2n-6, 20:3n-6, AA, DTA, DPA n-6); Σn-3 met = total n-3 metabolites = Σ(EPA, DPA n-3, DHA); Σn-3 = total n-3 FA = Σ(ALA, EPA, DPA n-3, DHA). AA, arachidonic acid; DTA, docosatetraenoic acid; DPA, docosapentaenoic acid; LA, linoleic acid; ALA, α-linolenic acid; GDM, gestational diabetes mellitus.
[b]Values marked with asterisks are significantly different from the control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.0001$.

TABLE 2
FA of Placental Ethanolamine Phosphoglycerides[a]

	Control (n = 25)		GDM (n = 11)	
	Median	25–75% IQR	Median[b]	25–75% IQR
16:0	7.67	7.32–8.14	5.91**	5.35–6.58
18:0	12.2	11.5–12.9	13.0	12.2–14.6
ΣSFA	20.0	19.1–21.0	18.8	18.4–19.8
16:1	0.54	0.45–0.64	0.13****	0.12–0.16
18:1	9.33	8.38–10.4	6.57****	5.68–7.45
ΣMono	10.1	9.03–11.1	6.95****	5.82–7.70
18:2n-6	6.22	5.26–7.51	4.00****	3.79–4.89
20:3n-6	3.33	2.96–3.80	2.94*	2.73–3.08
20:4n-6	24.3	23.4–26.3	25.7*	25.3–27.1
22:4n-6	2.29	2.18–2.66	3.36***	3.11–3.61
22:5n-6	1.51	1.30–1.70	1.80	1.18–2.18
Σn-6 met	32.7	30.6–34.9	34.5*	33.2–36.0
Σn-6	38.8	37.4–40.7	38.4	38.3–40.0
18:3n-3	0.06	0.05–0.07	Trace	Trace
20:5n-3	0.23	0.17–0.33	Trace	Trace
22:5n-3	1.49	1.34–1.77	1.92	1.28–2.83
22:6n-3	8.03	7.35–9.69	11.6***	9.80–11.9
Σn-3 met	10.3	8.95–11.5	13.3***	12.1–14.6
Σn-3	10.3	8.97–11.6	13.3***	12.2–14.6

[a]For abbreviations, see Table 1.
[b]Values marked with asterisks are significantly different from the control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.0001$.

TABLE 3
FA of Placental TG[a]

	Control (n = 25)		GDM (n = 11)	
	Median	25–75% IQR	Median[b]	25–75% IQR
16:0	25.8	25.2–29.6	28.3	27.1–29.2
18:0	8.99	8.00–11.1	9.11	5.35–9.67
ΣSFA	36.5	34.5–40.5	39.1	34.7–40.7
16:1	1.92	1.69–2.16	2.09	1.87–2.98
18:1	21.4	16.2–24.2	23.0	19.2–24.8
ΣMono	23.8	18.5–27.1	26.1	21.4–27.2
18:2n-6	11.0	9.31–15.8	15.6*	14.8–17.7
20:3n-6	3.98	3.28–4.60	2.48**	2.06–3.41
20:4n-6	9.58	8.31–11.6	7.38**	5.78–9.60
22:4n-6	1.28	1.03–1.43	0.90	0.66–1.03
22:5n-6	0.70	0.39–0.82	0.62	0.30–0.75
Σn-6 met	15.9	13.4–18.5	11.9**	9.79–15.9
Σn-6	26.9	25.2–33.0	29.4	26.7–30.8
18:3n-3	0.32	0.22–0.43	0.39	0.32–0.52
20:5n-3	0.37	0.26–0.48	0.32	0.18–0.43
22:5n-3	0.85	0.60–0.91	0.56**	0.36–0.70
22:6n-3	2.57	1.88–3.82	2.54	2.21–3.00
Σn-3 met	3.92	2.72–4.87	3.23	2.84–3.99
Σn-3	4.24	3.03–5.21	3.57	3.06–4.19

[a]For abbreviations, see Table 1.
[b]Values marked with asterisks are significantly different from the control group: *$P < 0.05$; **$P < 0.01$.

TG. In contrast to CPG and EPG, GDM had a negligible effect on placental TG FA composition. Nevertheless, compared with the controls, the GDM group had higher LA ($P < 0.05$), and lower DHGLA, AA, n-6 metabolites, and n-3 DPA ($P < 0.01$).

Ethnicity. There was no difference in the levels of placental AA and DHA in CPG, EPG, and TG between the Caucasian, African, and Asian subjects in the control group ($P > 0.05$).

DISCUSSION

In our previous communications, we reported that red cells of women with GDM at diagnosis (25), and plasma (24) and red cells (23) of their neonates at birth, had an abnormal FA composition. The two questions that arose from these studies were the following: (i) Is the abnormality a generalized phenomenon, or is it restricted to red cells of the mothers with GDM and plasma and red cells of their newborn babies? (ii) Why do the seemingly nondiabetic healthy babies of women with GDM have abnormal blood AA and DHA? The current study was undertaken to help address these questions.

The placenta is a very important tissue to investigate in relation to maternal-fetal AA and DHA status and balance. First, the human placenta is a vascular organ rich in phospholipids (28–30), which in turn are rich in PUFA, particularly AA (31). Second, the placenta lacks desaturase activity (13), and is thought to be primarily dependent on maternal circulation for the supply of these vital FA. Consequently, diabetes-induced abnormality in supply and/or uptake would be expected to alter placental AA and DHA composition.

In contrast to plasma (26) and red cells (25), the placenta of the women diagnosed with GDM had higher levels of both AA and DHA compared with the control subjects. The AA level was elevated by 11 and 6%, and the DHA level by 38% and 29%, respectively, in CPG and EPG of the diabetics. These differences between the two groups were unlikely to have been a reflection of their dietary background because the level of AA was reduced by 30% and the level of DHA was unaltered in placental TG of the GDM relative to that of the control group.

The GDM group comprised four Africans and seven Asians, and the control group comprised six Caucasians, six Africans, and 13 Asians. It is conceivable that the observed difference between the two groups could be due to the ethnic imbalance of the populations studied. However, the difference in the pattern of the major FA in CPG, EPG, and TG between the GDM and control Asian women was similar to that of the total GDM and control groups. In addition, there was no difference in either of the two FA between the Asian, African, and Caucasian subjects in the control group. This is consistent with our previous study (31), which showed that ethnicity had no significant effect on placental AA and DHA.

There are no published data on placental FA of women with GDM. However, the placentas of women with type 1 diabetes have been reported to have reduced DHA and normal AA levels compared with those of nondiabetics (32). The contrast between our findings and those of Lakin and colleagues (32) could be a reflection of the two different disease entities. In the latter study, FA of placental total lipids (phospholipids and TG), rather than of the individual lipid fractions, were determined. Hence, it is conceivable that the real effect of diabetes on the FA composition of the individual phospholipids might have been masked by the disproportionate contribution of the neutral lipids, primarily TG.

The higher levels of AA and DHA in CPG and EPG of the GDM group could not be explained by enhanced synthesis because the placenta is thought to be devoid of desaturase activity (13). As maternal circulation is the primary source of placental AA and DHA, our data suggest that the uptake of these two FA is enhanced in gestational diabetes. Consistent with this proposition, there is evidence of alteration of the transporters and transport of long-chain PUFA (LCPUFA) in experimental animals (33,34), a higher incorporation of AA into TG of placental tissue in insulin-dependent diabetes mellitus pregnancy (35), and enhanced expression of the liver FA-binding protein in placental homogenates of women with GDM (36). Indeed, Bonen *et al.* (37) hypothesized that the intramuscular accumulation in human obesity and type 2 diabetes is attributable to an increased rate of LCPUFA transport as the result of an increase in the FA transporters, FA translocase (FAT/CD36), and/or plasma-membrane–associated FA-binding protein (FABPpm).

The lower level of AA in the placental TG in contrast to that in CPG and EPG in the GDM group could indicate that the TG AA was selectively transferred to the fetus. Bonet *et al*. (38) showed a 10-fold preference in uptake of FA from TG compared with uptake of albumin-bound FA by isolated placental trophoblasts. Also, it could be that the TG was used for eicosanoid production (39), hydrolyzed and re-esterified into phosphoglycerides, or both.

The question is whether the abnormal FA composition of the placenta of the women in the GDM group, which was manifested by higher levels of AA and DHA in CPG and EPG, would help explain the reduced levels of the two FA in the newborn offspring of women with GDM reported by Min *et al.* (23) and Thomas *et al.* (24). The fetus and neonate are able to synthesize AA and DHA (14–17). However, because of the slow rate of synthesis and the consequent inability to support optimal development (18,19), it is acknowledged that maternal circulation is the major source of AA and DHA for the fetus. The enhanced levels of the two FA in the placenta and the concomitant reduction of levels in the fetus suggest that the two FA are taken up by the placenta and retained after esterification into phosphoglycerides instead of being transferred to the fetus. The amount retained is likely to be significant because, in the placenta, phospholipids account for about 80% of total lipids, EPG and CPG for 60% of the phospholipids, and LCPUFA for 40% of the phospholipid FA (28–30).

The limitations of this study were the ethnic heterogeneity of the subjects and the lack of FA intake data of the mothers. Nevertheless, in spite of these shortcomings, the investigation reveals that, in contrast to fetal blood, the placentas of women with pregnancy-induced transient diabetes mellitus exhibit elevated levels of the vital FA AA and DHA. The mechanism involved and the effect of this phenomenon on long-term fetal growth and development remain to be elucidated.

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Effect of Diacylglycerol on the Development of Impaired Glucose Tolerance in Sucrose-Fed Rats

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ABSTRACT: The effects of DAG oil and TAG oil on impaired glucose tolerance in rats that were fed a diet containing high levels of sucrose were compared. Male Wistar rats (8 wk old and 32 wk old) were fed either high-sucrose (57.5% sucrose w/w) or control diets containing either 10% (w/w) DAG or TAG oil with a similar FA composition for 48 wk in 8-wk-old rats and for 24 wk in 32-wk-old rats. Plasma lipids, the size of the islets of Langerhans, and insulin, glucose, and adipocytokine levels were measured. An oral glucose tolerance test (OGTT) was carried out during the study period. For rats in both age groups that were fed a high-sucrose diet, the DAG oil group had lower plasma glucose and insulin response in an OGTT, and lower homeostasis model assessment-R levels, than the TAG oil group. Furthermore, in 8-wk-old rats that were fed a high-sucrose diet, the DAG oil group accumulated less visceral fat and showed decreases of plasma adiponectin and suppressed increases of plasma insulin, leptin, and the size of islet of Langerhans compared with the TAG oil group. No difference in the OGTT was found between the DAG and TAG oil groups in either age group of rats fed a control diet. In conclusion, these results suggest that DAG oil ingestion prevents the high-sucrose-diet–induced development of impaired glucose tolerance compared with TAG oil ingestion.

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In industrialized societies, type 2 diabetes is a common cause of morbidity and mortality. The disease is characterized by impaired glucose tolerance and insulin resistance, which is frequently accompanied by visceral fat obesity (1–3). The typical Western diet is very high in fat and sucrose and is considered to be a major factor in the development of impaired glucose tolerance, insulin resistance, and visceral fat obesity. Foods that are designed to prevent and reduce visceral fat obesity may help to avoid diet-induced diabetes characterized by impaired glucose tolerance and insulin resistance.

DAG, a natural component of various edible oils, contains only two esterified FA chains, in contrast to the three FA chains in TAG. DAG oil is an edible oil containing approximately 80–90% DAG. Most of the DAG (70%) is the 1,3-DAG isoform, and its energy value is practically the same as ordinary oil containing TAG (4–6). Results of studies in animals and humans have revealed that DAG oil has metabolic characteristics that are distinct from those of TAG oil with a similar FA composition, and that these characteristics can be beneficial in preventing and managing the accumulation of visceral fat and the postprandial elevation of triglyceride in serum and chylomicrons (4,5,7–16). In addition, the long-term ingestion of DAG oil instead of TAG oil decreases the serum triglyceride and glycohemoglobin A_{1c} (HbA_{1c}) levels in type 2 diabetic patients with hypertriglyceridemia. It also suppresses the increase in fasting blood glucose and insulin levels in high-fat, high-sucrose-diet–fed C57BL/6J mice, and in blood glucose levels in an oral glucose tolerance test (OGTT) in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (9,10,17,18). These results suggest that the ingestion of DAG oil not only prevents the accumulation of visceral fat but also suppresses the development of abnormal carbohydrate metabolism.

Impaired glucose tolerance is accompanied by visceral fat accumulation that develops in normal rats fed sucrose instead of starch as a carbohydrate source, and depends on both the amount of sucrose and the length of time for which it is used in the diet (19–25). In addition, impaired glucose tolerance is also induced with aging (26,27). The objectives of this study were to investigate the effect of the long-term ingestion of DAG oil, in comparison with TAG oil, on impaired glucose tolerance as well as on visceral fat accumulation in high-sucrose-diet–fed young (8-wk-old) and old (32-wk-old) rats.

EXPERIMENTAL PROCEDURES

Test oils. The DAG oil was prepared by esterifying glycerol with FFA derived from rapeseed oil and soybean oil by the method of Huge-Jensen *et al.* (28). The TAG oil was blended from rapeseed oil, safflower oil, and perilla oil. The FA composition of both oils is shown in Table 1. The FA composition of the DAG oil was very similar to that of the TAG oil. The DAG concentration of the DAG oil was 85.01/100 g and the ratio of 1(3),2- to 1,3-DAG was 34.5:65.5.

Animals and experimental design. Male Wistar rats (8 wk old and 32 wk old) were purchased from CLEA Japan (Tokyo, Japan). They were maintained in a temperature-controlled environment (23 ± 2°C) on a 12-h light/dark cycle. After an acclimatization period of 7 d, each group (8-wk-old or 32-wk-old) of rats was divided into four groups (n = 6/group), so that the body weight of each group was approximately equal, and the rats were transferred to individual cages. The 8-wk-old and

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Abbreviations: EIA, enzyme-linked immunosorbent assay; HbA_{1c}, glycohemoglobin A_{1c}; HOMA-R, homeostasis model assessment-R; NEFA, nonesterified FA; OGTT, oral glucose tolerance test; OLETF, Otsuka Long-Evans Tokushima Fatty; SD, Sprague-Dawley.

TABLE 1
FA Compositions of Test Oils[a]

FA	TAG	DAG
C14	0.1	—
C16	5.4	3.1
C16:1	0.2	—
C18	2.0	1.2
C18:1	37.1	39.2
C18:2	46.0	47.5
C18:3	7.3	8.6
C20	0.5	0.2
C20:1	0.9	0.2
C22	0.2	—
C22:1	0.1	—
TOTAL	99.7	100.0

[a]Percentage by weight.

32-wk-old rats were fed with control or a high-sucrose diet including 10% TAG or DAG oil, respectively (Table 2). The 8-wk-old and 32-wk-old rats were maintained on these diets for 48 and 24 wk, respectively. All rats were allowed access to water and food ad libitum. The energy values for each diet were calculated from the macronutrient composition using values of 4 kcal/g, 4 kcal/g, 9.3 kcal/g, and 9.2 kcal/g for carbohydrate, protein, DAG oil, and TAG oil, respectively. Mesenteric, epididymal, perirenal, and retroperitoneal white adipose tissues as visceral fat, as well as the liver and pancreas, were dissected from each rat at the end of the study period. The food intake for each rat was recorded every 3–4 d. Body weights of all rats were recorded at weekly intervals. Animal experiments were performed with the approval of the Ethics Committee for Experimental Animals of the Kao Corporation.

Plasma lipids and adipocytokines. At weeks 0, 20, and 44 after initiation of the experiment for the 8-wk-old rats, and at weeks 0 and 20 after initiation of the experiment for the 32-wk-old rats, blood samples were withdrawn from the jugular vein after an 18-h fast. Plasma was obtained by centrifugation at 1500 × *g* for 15 min at 4°C. Plasma triglyceride and nonesterified FA (NEFA) levels were determined enzymatically using L-type Wako TG-H and NEFA-HA tests, respectively (Wako, Osaka, Japan). Plasma leptin and adiponectin were measured by means of a leptin enzyme-linked immunosorbent assay (EIA) kit (Morinaga, Yokohama, Japan) and an adiponectin EIA kit (Otsuka, Tokushima, Japan).

OGTT. At weeks 5, 9, 13, 21, 29, and 45 after initiation of the experiment for the 8-wk-old rats, and at weeks 5, 9, 13, and 21 after initiation of the experiment for the 32-wk-old rats, rats were fasted for 20 h and blood samples were taken from the tail vein for the determination of fasting glucose and insulin levels. An oral glucose challenge (2 g/kg) was administered to unrestrained animals, and blood samples were taken at 30, 60, 90, and 120 min after glucose ingestion. Blood glucose was measured using ACCU-CHEK Comfort (Roche Diagnostics, Basel, Switzerland). Plasma insulin was measured by means of a rat insulin EIA kit (Morinaga). The homeostasis model assessment-R (HOMA-R) was estimated using the following formula: HOMA-R = fasting insulin (μU/mL) × fasting glucose (mg/dL)/405.

Size of islet of Langerhans in pancreas. At the end of the study period, the pancreas was rapidly removed, washed with saline, fixed in 10% buffered formalin, and embedded in paraffin. Each paraffin-embedded block was sectioned and stained with Gomori's aldehyde fuchsin trichrome (29). Each stained section was photographed with an Olympus DP70 camera (Olympus Co. Ltd., Tokyo, Japan). The number of islets was counted and the diameter of islets was measured using Scion Image (Scion Co. Ltd., Frederick, MD).

Statistical analysis. The data are represented as the means ± standard deviation, and statistical differences in body weight, visceral fat weight, liver weight, energy intake, plasma lipid level, plasma adipocytokine level, plasma insulin level, blood glucose level, HOMA-R level, and islet diameter between the groups were determined using the student t-test. Repeated measures ANOVA was used to investigate changes in blood glucose, plasma insulin, and HOMA-R over time and in the OGTT. A statistical model was constructed using the effect of oil, time, and the interaction of oil × time. In all analyses, a significant difference was defined as $P < 0.05$. These statistical calculations were

TABLE 2
Nutrient Composition of Experiment Diets in Rats

	Dietary group (g/100 g diet)			
	Control		High-sucrose	
Ingredient	TAG	DAG	TAG	DAG
TAG oil	10.0	—	10.0	—
DAG oil	—	10.0	—	10.0
α-Potato starch	61.0	61.0	—	—
Sucrose	—	—	57.5	57.5
Casein	20.0	20.0	20.0	20.0
Cellulose	4.0	4.0	7.5	7.5
Mineral mix[a]	4.0	4.0	4.0	4.0
Vitamin mix[b]	1.0	1.0	1.0	1.0
kcal/100 g	411.1	409.7	425.4	424.0

[a]AIN-76 prescription.
[b]AIN-76 prescription + choline bitartrate (20g/100g).

TABLE 3
Visceral Fat and Liver Weights[a] in 8-Wk-Old Rats and 32-Wk-Old Rats[b] Which Fed with Control or High-Sucrose Diet Containing TAG or DAG Oil

		Control		High-Sucrose	
		TAG	DAG	TAG	DAG
8-wk-old rats	Visceral fat weight (g)				
	Epididymal	12.0 ± 2.2	11.0 ± 2.2	13.5 ± 2.7	11.5 ± 1.9
	Retroperitoneal	18.9 ± 3.4	16.9 ± 3.7	24.2 ± 3.1	20.5 ± 1.3[c]
	Perirenal	4.7 ± 0.6	4.1 ± 0.8	5.0 ± 0.7	4.7 ± 0.8
	Mesenteric	14.2 ± 3.0	12.2 ± 2.5	17.0 ± 2.3	15.2 ± 1.5
	Total	49.9 ± 8.0	44.3 ± 7.8	59.3 ± 4.6	52.0 ± 4.0[c]
	Liver weight (g)	11.5 ± 1.1	10.9 ± 1.1	12.5 ± 1.3	12.9 ± 0.9
	Visceral fat weight (g) 32-wk-old rats				
	Epididymal	9.9 ± 1.3	10.7 ± 1.4	12.6 ± 2.8	12.2 ± 1.9
	Retroperitoneal	15.6 ± 2.6	17.3 ± 1.8	25.7 ± 6.2	22.2 ± 4.5
	Perirenal	3.3 ± 0.7	3.6 ± 0.6	4.6 ± 0.5	4.3 ± 0.7
	Mesenteric	11.2 ± 1.6	11.6 ± 1.6	16.9 ± 3.1	15.1 ± 1.2
	Total	40.0 ± 4.7	43.1 ± 3.7	59.7 ± 10.2	53.8 ± 6.7
	Liver weight (g)	12.1 ± 0.9	12.1 ± 1.1	13.9 ± 0.9	13.8 ± 1.1

[a]Values are means ± SD (n = 6/group).
[b]Rats were sacrificed, at the end of the study period, after 48 wk and 24 wk feeding with each diet in 8-wk-old and 32-wk-old rats, respectively.
[c]$P < 0.05$ vs. TAG oil group.

performed with StatView for Windows, version 5.0 (SAS Institute, Cary, NC).

RESULTS

Energy intake, body weight, and general observation. In 8-wk-old rats, the average energy intake during the study period in groups fed the control diet with TAG oil, the control diet with DAG oil, the high-sucrose diet with TAG oil, and the high-sucrose diet with DAG oil was 68.15 ± 4.17, 64.58 ± 4.16, 69.79 ± 2.87, and 67.73 ± 2.98 kcal/d/rat, respectively. In 32-wk-old rats, the average energy intake during the study period in groups fed the control diet with TAG oil, the control diet with DAG oil, the high-sucrose diet with TAG oil, and the high-sucrose diet with DAG oil was 71.7 ± 3.81, 68.69 ± 2.01, 79.45 ± 5.87, and 77.45 ± 6.06 kcal/d/rat, respectively. No differences were found in energy intake between the TAG and DAG oil groups. In addition, time-course changes in body weight in 8-wk-old rats and 32-wk-old rats that were fed control or high-sucrose diets containing TAG or DAG oil during the study period were measured at weekly intervals; no differences were found between the TAG and DAG oil groups. All of the rats remained healthy during the study period.

Visceral fat and liver weight. The visceral fat (perirenal, retroperitoneal, mesenteric, and epididymal fat) weight and the liver weight in the rats in each group of this study are shown in Table 3. In the 8-wk-old rats fed with a high-sucrose diet, total visceral fat weight was lower in the DAG oil group compared to that in the TAG oil group, whereas no difference in total visceral fat weight was noted between the DAG and TAG oil groups in the 32-wk-old rats fed with a high-sucrose diet. The liver weight was similar between the DAG and TAG oil groups. In rats of either age group fed a control diet, no differences in the total visceral fat weight or liver weight was found between the DAG and TAG oil groups.

Plasma lipids and adipocytokines. Plasma lipid and adipocytokine levels in the rats in each group of this study are shown in Table 4. In the 8-wk-old rats fed a high-sucrose diet, the elevation in triglyceride and leptin levels were suppressed at week 20 and the reduction in adiponectin levels was suppressed at week 44 in the DAG oil group compared to those in the TAG oil group. In contrast, in the 32-wk-old rats fed a high-sucrose diet, no differences were noted in the plasma lipid and adipocytokine levels between the DAG and TAG oil groups. In both 8-wk-old and 32-wk-old rats fed a control diet, no differences in plasma lipid and adipocytokine levels were found between the DAG and TAG oil groups.

Glucose, insulin, and HOMA-R. Time-course changes in fasting blood glucose levels, plasma insulin levels, and HOMA-R levels in the rats in each group of this study are shown in Figure 1. In the 8-wk-old rats fed a high-sucrose diet, the elevation in fasting plasma insulin and HOMA-R levels were suppressed in the DAG oil group compared to those in the TAG oil group. In addition, in the 32-wk-old rats fed a high-sucrose diet, the HOMA-R level was lower in the DAG oil group compared to that in the TAG oil group.

OGTT. Time-course changes in blood glucose and plasma insulin levels in an OGTT at weeks 5, 9, 13, 21, 29, and 45 after initiation of the experiment in the case of the 8-wk-old rats, and at weeks 5, 9, 13, and 21 after initiation of the experiment in the case of the 32-wk-old rats, for each group of this study, are shown in Figure 2. In the 8-wk-old rats fed a high-sucrose diet, the elevation in blood glucose levels was suppressed at weeks 9, 13, and 21, and the elevation in plasma insulin levels was

TABLE 4
Plasma Lipid and Adipocytokine Levels[a] in 8-Wk-Old Rats and 32-Wk-Old rats[b] Fed with Control or High-Sucrose Diet Containing TAG or DAG oil

			Control		High-Sucrose	
			TAG	DAG	TAG	DAG
8-wk-old rats	Triglyceride					
	(mg/dL)	0	67.9 ± 22.4	65.6 ± 21.7	69.7 ± 10.5	71.3 ± 21.5
		20	104.3 ± 9.7	110.7 ± 31.7	151.6 ± 25.4	104.3 ± 26.5[c]
		44	138.1 ± 21.0	125.7 ± 57.0	146.8 ± 17.9	141.9 ± 19.6
	NEFA					
	(mEq/L)	0	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.2
		20	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.6 ± 0.2
		44	0.8 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.2
	Leptin					
	(ng/mL)	0	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	0.2 ± 0.1
		20	3.1 ± 1.0	2.6 ± 0.8	5.2 ± 2.0	2.5 ± 1.0[c]
		44	5.3 ± 1.6	4.7 ± 1.3	8.2 ± 1.8	6.3 ± 0.9
	Adiponectin					
	(μg/mL)	0	4.4 ± 0.6	4.4 ± 0.6	4.1 ± 0.9	4.3 ± 0.7
		20	6.2 ± 0.7	6.3 ± 0.7	4.8 ± 0.6	5.3 ± 0.6
		44	7.1 ± 0.8	7.0 ± 0.6	6.0 ± 0.5	6.8 ± 0.6[c]
32-wk-old rats	Triglyceride					
	(mg/dL)	0	71.7 ± 18.8	69.8 ± 21.3	70.0 ± 14.9	65.1 ± 8.7
		20	114.4 ± 21.8	114.3 ± 35.2	190.8 ± 46.1	157.9 ± 53.3
	NEFA					
	(mEq/L)	0	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2
		20	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
	Leptin					
	(ng/mL)	0	1.1 ± 0.5	1.5 ± 0.5	1.6 ± 0.7	1.6 ± 0.7
		20	3.9 ± 0.5	4.7 ± 1.7	7.1 ± 1.9	5.5 ± 1.8
	Adiponectin					
	(μg/mL)	0	5.3 ± 0.7	5.7 ± 0.8	4.9 ± 0.9	5.2 ± 1.1
		20	5.9 ± 1.2	5.7 ± 1.1	5.3 ± 2.0	6.0 ± 1.6

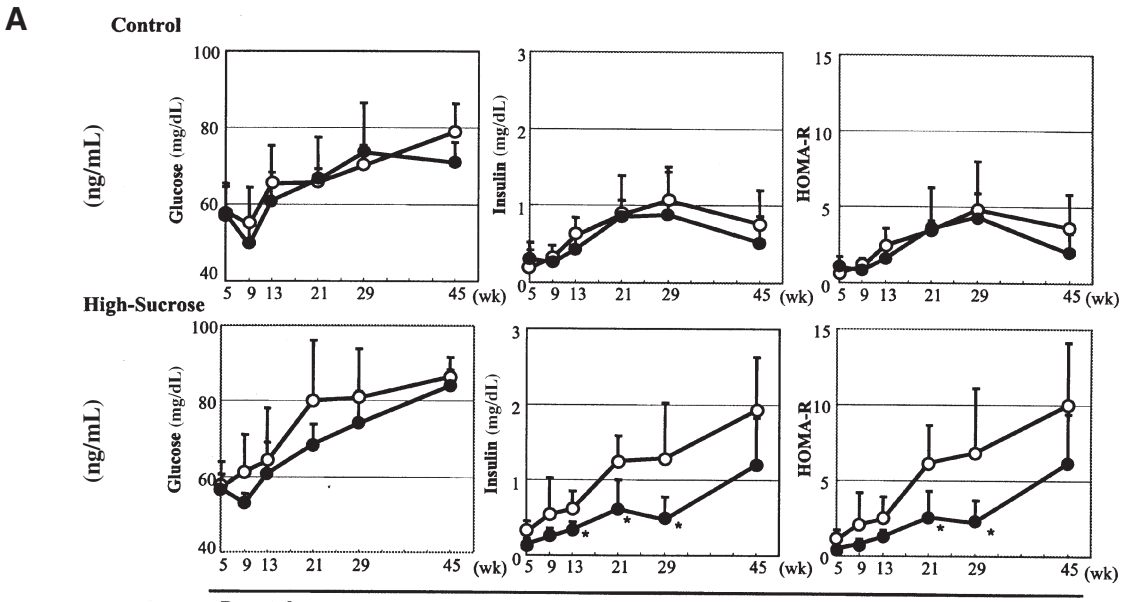
[a]Values are means ± SD (n = 6 / group).
[b]Rats were sacrificed, at the end of the study period, after 48 wks and 24 wks feeding with each diet in 8-wk-old and 32-wk-old rats, respectively.
[c]$P < 0.05$ vs TAG oil group. NEFA, nonesterified FA.

suppressed at weeks 13, 21, and 29 in the DAG oil group compared to the TAG oil group. In the 32-wk-old rats fed a high-sucrose diet, the elevation in blood glucose levels was suppressed at week 5, and the elevation in plasma insulin levels was suppressed at weeks 5 and 21 in the DAG oil group compared to the TAG oil group. However, in rats in either age group fed a control diet, no differences were noted between the DAG and TAG oil groups.

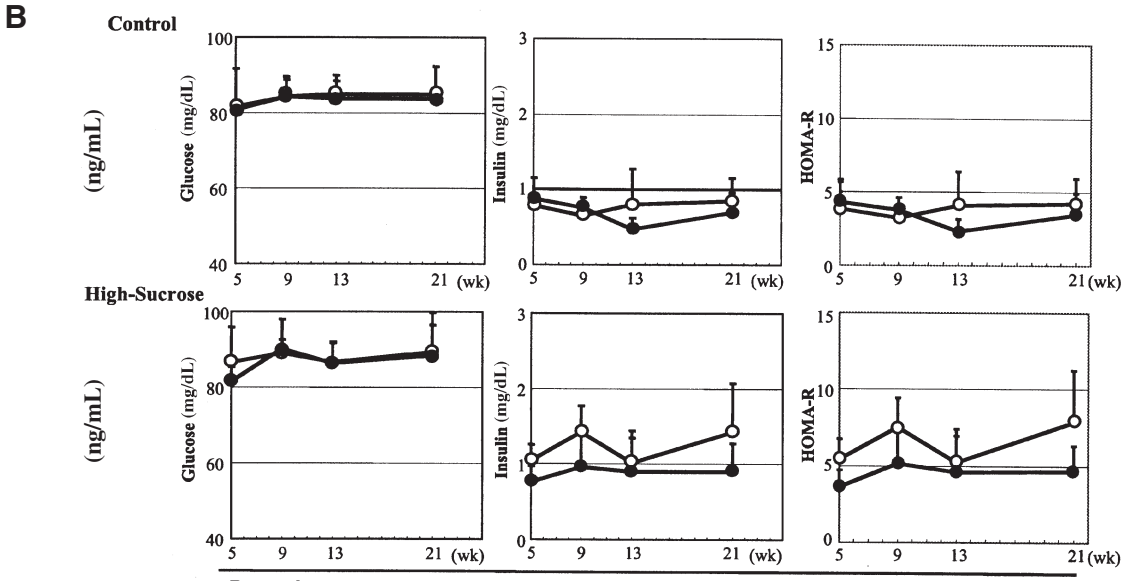
Size of islets of Langerhans in pancreas. The distributions of size of the islets of Langerhans in the pancreas in the rats in each group of this study are shown in Figure 3. In the 8-wk-old rats fed a high-sucrose diet, smaller islets appeared more frequently and the average diameter of the islets was smaller in the DAG oil group compared to the TAG oil group. In contrast, in the 32-wk-old rats fed a high-sucrose diet and either 8-wk-old or 32-wk-old rats fed a control diet, no differences were noted in the distribution and islet diameter between the DAG and TAG oil groups.

DISCUSSION

The accumulation of visceral fat is associated with the development of hyperinsulinemia and insulin resistance (19,23,30–32). The replacement of TAG oil with DAG oil in the diet was found to suppress the accumulation of visceral fat and the elevation in plasma insulin levels in 8-wk-old rats fed a high-sucrose diet. Murase *et al.* reported that the amount of visceral fat and the insulin levels were increased in C57BL/6J mice fed a high-fat, high-sucrose diet, compared to mice fed a control diet, but the replacement of TAG oil with DAG oil in the high-fat, high-sucrose diet suppressed these increases (9,10). Muzumdar *et al.* reported that the age-related elevation of the plasma insulin levels was suppressed by the surgical removal of visceral fat and by limitations in food intake in Sprague-Dawley (SD) rats (33). Thus, DAG oil suppressed the high-sucrose-diet–induced increase in plasma insulin levels, and this effect may have been due to the suppression of visceral fat accumulation as the result of DAG oil ingestion. In addition, the replacement of TAG oil with DAG oil in the diet suppressed the high-sucrose-diet–induced increase in HOMA-R levels. HOMA-R levels are a known index of insulin resistance (34–36). Furthermore, the use of DAG oil suppressed the high-sucrose-diet–induced increase in plasma leptin at week 20 in 8-wk-old rats compared with the TAG oil group. In obese individuals, the elevation of plasma leptin levels induces leptin resistance, and this event is generally thought to trigger insulin resistance (37). These results suggest



Repeated measures ANOVA	Control			High-Sucrose		
	Glucose	Insulin	HOMA-R	Glucose	Insulin	HOMA-R
Oil	NS	NS	NS	NS	S	S
Time	S	S	S	S	S	S
Oil x Time	NS	NS	NS	NS	NS	NS

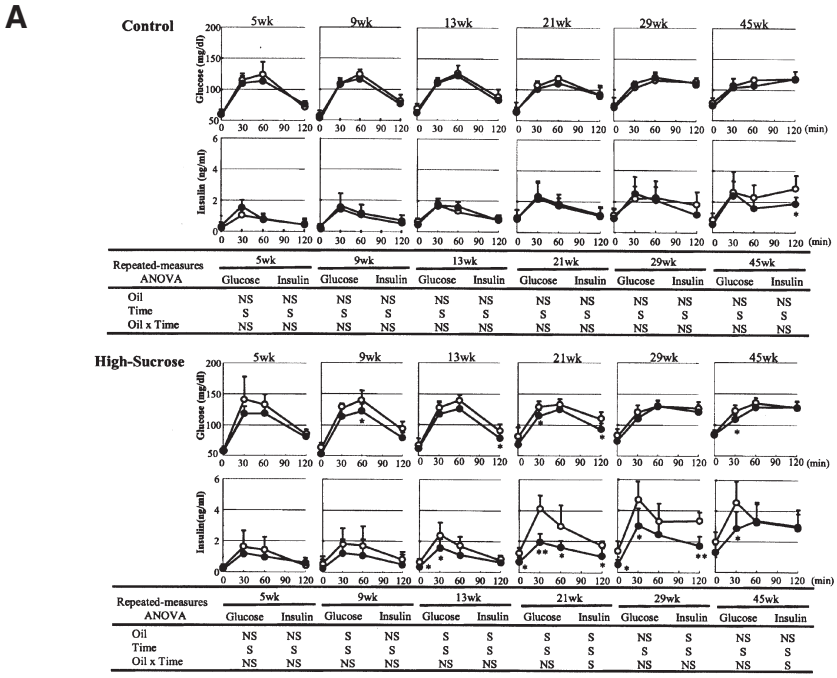


Repeated measures ANOVA	Control			High-Sucrose		
	Glucose	Insulin	HOMA-R	Glucose	Insulin	HOMA-R
Oil	NS	NS	NS	NS	NS	S
Time	S	S	S	S	S	S
Oil x Time	NS	NS	S	NS	NS	NS

FIG. 1. Time-course changes in fasting blood glucose, plasma insulin, and HOMA-R levels in 8-wk-old rats (A) and 32-wk-old rats (B) fed control or high-sucrose diets containing TAG or DAG oil. Closed circles indicate DAG oil and open circles indicate TAG oil. Values are means ± SD (n = 6/group). A statistical model of the repeated measures ANOVA was constructed using the effect of oil, time, and interaction of oil × time: effect significant, S ($P < 0.05$), or not significant, NS. *$P < 0.05$ versus TAG oil group. HOMA-R: fasting insulin (μU/mL) × fasting glucose (mg/dL)/405.

that DAG oil might prevent the high-sucrose-diet–induced development of hyperinsulinemia and insulin resistance compared to TAG oil.

Compared with TAG oil, DAG oil suppressed the elevation in blood glucose levels in an OGTT in rats fed a high-sucrose diet, suggesting that DAG oil prevents the development of impaired glucose tolerance. However, this effect was not noted at week 29 (at age 37 wk) or later in the 8-wk-old rats, or at week



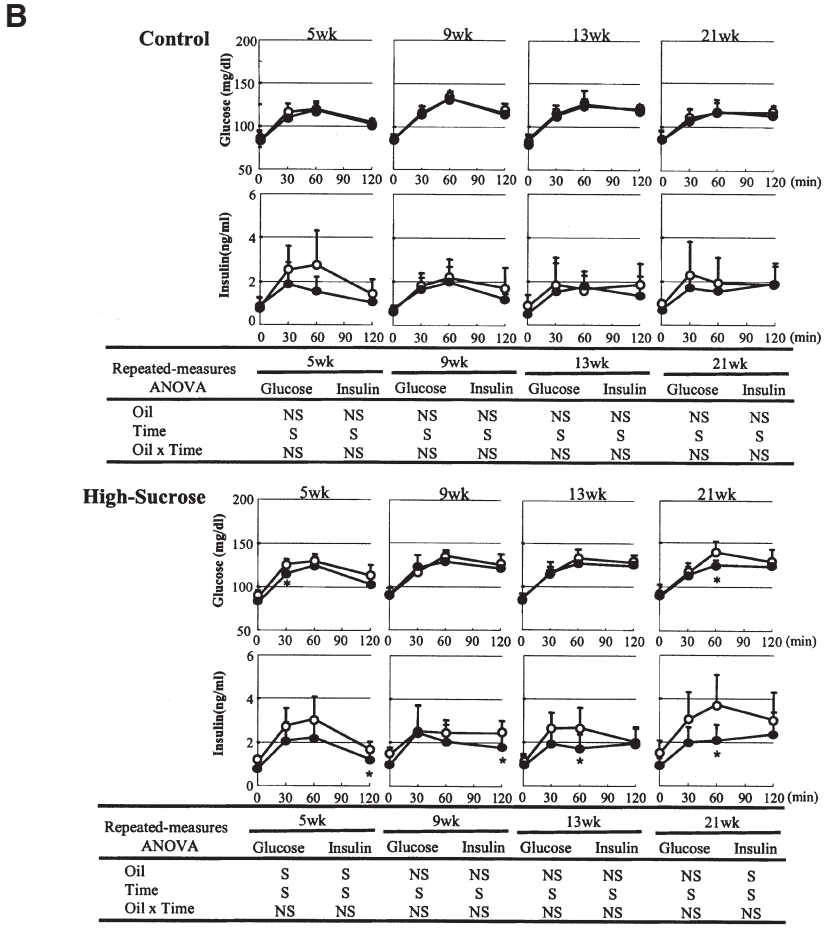


FIG. 2. Time-course changes in blood glucose and plasma insulin levels in an oral glucose tolerance test (OGTT) at weeks 5, 9, 13, 21, 29, and 45 after initiation of the experiment in the 8-wk-old rats (A) and at weeks 5, 9, 13, and 21 after initiation of the experiment in the 32-wk-old rats (B) fed control or high-sucrose diets containing TAG or DAG oil. Closed circles indicate DAG oil and open circles indicate TAG oil. Values are means ± SD (n = 6/group). A statistical model of the repeated measures ANOVA was constructed using the effect of oil, time, and interaction of oil × time: effect significant, S ($P < 0.05$), or not significant, NS. *$P < 0.05$ versus TAG oil group. **$P < 0.01$ versus TAG oil group.

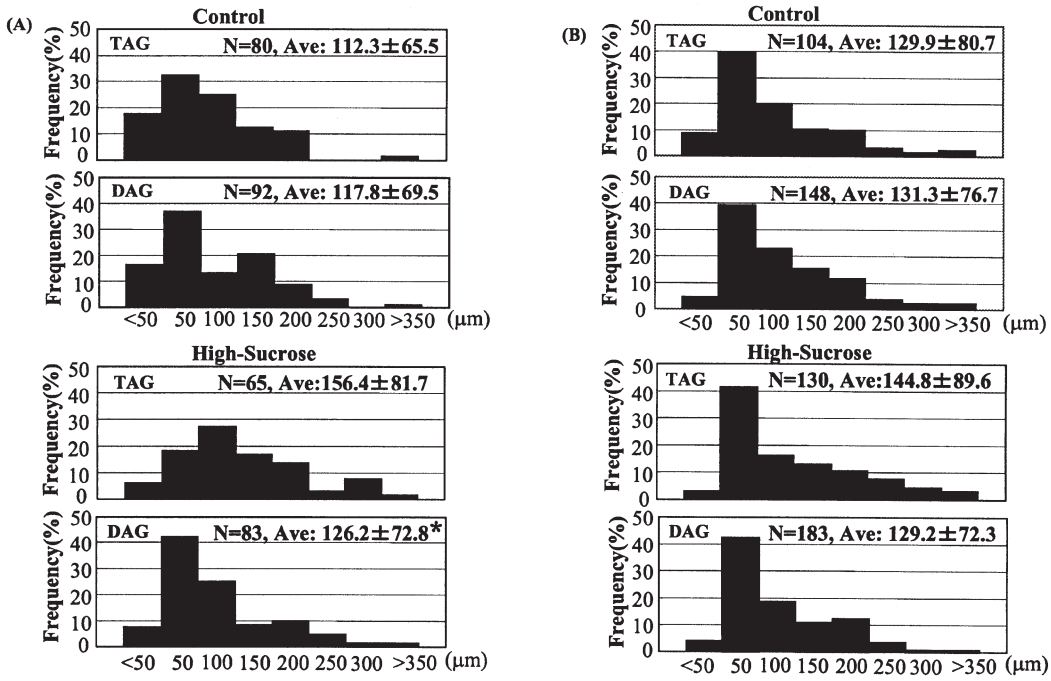


FIG. 3. The size distribution of islets of Langerhans in the pancreas in 8-wk-old rats (A) and 32-wk-old rats (B) fed control or high-sucrose diets including TAG or DAG oil. The distribution of size of islet (μm) is represented every 50 μm diameter size. *N*, total number of islets observed (n = 6/group); ave., the average diameter size (μm) observed for all islets; values represented as means ± SD. *$P < 0.05$ versus TAG oil group.

9 (at age 41 wk) or later in the 32-wk-old rats, and the reason for this is not clear. The ingestion of a high-sucrose diet is known to lead to impaired glucose tolerance in normal rats (21,24,25). In addition, impaired glucose tolerance is also induced by aging (26,27). Fink *et al.* reported that serum insulin and blood glucose levels were increased in elderly subjects (69 ± 1 yr old), compared to young subjects (37 ± 2 yr old) in an OGTT, and that this caused a decrease in peripheral glucose disposal with aging (38). Matthaei *et al.* reported that insulin-stimulated glucose incorporation in rat adipocytes was suppressed with aging (39). In the current study, the suppression of the development of impaired glucose tolerance by DAG oil ingestion disappeared at the age of 37 wk and 41 wk in 8-wk-old rats and 32-wk-old rats, respectively. Therefore, the promotion of impaired glucose tolerance with aging might have a stronger influence than the DAG oil–induced suppression of impaired glucose tolerance.

Murata *et al.* reported that when SD rats were fed a DAG or TAG oil diet for 14 d, the activities of β-oxidation-related enzymes in the liver, acyl-CoA dehydrogenase, acyl-CoA oxidase, enoyl-CoA hydrase, and 3-hydroxyacyl-CoA dehydrogenase, were increased with the DAG oil additive dose (40). Murase *et al.* also reported that DAG oil with high-fat, high-sucrose-diet–fed C57BL/6J mice, compared with TAG oil, increased the expression of lipid metabolism–related genes and β-oxidation activity in the liver and small intestine (9,10). These findings suggest that the effect of the suppression of visceral fat accumulation in 8-wk-old rats fed a high-sucrose diet containing DAG oil might be due to DAG oil activating more β-oxidation-related enzymes in the liver and small intestine compared with TAG oil. On the other hand, in this study, the use of DAG oil suppressed the high-sucrose-diet–induced decrease in the plasma adiponectin levels at week 44 in 8-wk-old rats, compared to TAG oil. A decrease in plasma adiponectin levels is accompanied by obesity and diabetes (41–44). It has been reported that adiponectin regulates glucose and lipid metabolism and activates energy expenditure by activating AMP kinase (45,46). Furthermore, Fruebis *et al.* reported that adiponectin treatment induced weight loss without decreasing food intake in mice consuming a high-fat, high-sucrose diet (47). Thus, the suppression of the decrease in plasma adiponectin levels in 8-wk-old rats fed a high-sucrose diet containing DAG oil compared with those fed a high-sucrose diet containing TAG oil may also contribute to the suppression of visceral fat accumulation by the activation of energy expenditure.

In this study, not only plasma analysis but also histological analysis has been conducted to accurately verify the effect of DAG oil on the development of high-sucrose-diet–induced hyperinsulinemia and impaired glucose tolerance. DAG oil suppressed the high-sucrose-diet–induced increase in the size of islets of Langerhans in the pancreas, compared to TAG oil. Huo *et al.* (48) reported that the development of insulin resistance in C57BL/6J mice fed a high-sucrose, high-fat diet induced an increase in the size of the islets of Langerhans in the pancreas compared with mice fed a control diet. In this study, in 8-wk-

old rats fed the high-sucrose diet, a reduction in blood glucose levels after an OGTT was observed at weeks 9, 13, and 21 in the DAG oil group compared to those in the TAG oil group, as shown in Figure 2A. This finding suggests that DAG oil prevents the high-sucrose-diet–induced development of impaired glucose tolerance. Hypertrophy of islet size in the high-sucrose diet was reduced in the DAG oil group versus the TAG oil group, perhaps because blood glucose levels are better regulated with less hyperinsulinaemia. In addition, in rats fed the high-sucrose diet, the suppression of visceral fat accumulation was observed in the DAG oil group versus the TAG oil group. Ikeda *et al.* (49) reported that obesity was readily induced, hyperglycemia and hyperinsulinemia developed, and the size of the islets of Langerhans in the pancreas significantly increased in obese-hyperglycemic Wistar fatty rats compared with lean Wistar rats. Thus, the suppression of the high-sucrose-diet–induced increase in the size of islets by DAG oil ingestion, compared with TAG oil ingestion, may have been due to the suppression of visceral fat accumulation and of the development of hyperinsulinemia and impaired glucose tolerance.

Sugimoto *et al.* reported the possibility that the replacement of ordinary oil in the diet with DAG oil induced an impaired glucose tolerance in normal rats and Wistar fatty rats that were fed a high-sucrose diet for 12 and 5 wk, respectively (50,51). The findings herein were not consistent with the results reported by Sugimoto *et al.*, and the reason for the discrepancy is not clear. Yamamoto *et al.* reported that the ingestion of DAG oil for 12 wk by type 2 diabetic patients with hypertriglyceridemia decreased the serum HbA_{1c} levels, compared with patients who ingested TAG oil (17). In an animal study, Mori *et al.* (18) reported that the long-term consumption of DAG oil instead of TAG oil in OLETF rats suppressed the increase in blood glucose levels in an OGTT. Murase *et al.* also reported that DAG oil suppressed the high-fat, high-sucrose-diet–induced increase in blood glucose and insulin levels compared with C57BL/6J mice that were fed TAG oil (9,10). These reports suggest that DAG oil consumption might suppress the development of abnormal carbohydrate metabolism, consistent with the results reported in this study. Further detailed experiments might be required to clarify and validate this conclusion.

In this study, we demonstrated that the ingestion of DAG oil suppressed the high-sucrose-diet–induced increase in blood glucose and plasma insulin response in an OGTT, and in HOMA-R levels, compared with TAG oil ingestion. These findings suggest the possibility of the prevention and improvement of impaired glucose tolerance and insulin resistance via the use of DAG oil.

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Rat Adult Offspring Serum Lipoproteins Are Altered by Maternal Consumption of a Liquid Diet

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ABSTRACT: Palatable liquid diets for the administration of ethanol (EtOH) to animals have proven to be a major advance for the study of the effects of EtOH consumption under conditions of isocaloric nutrition of the control animals. Using a liquid diet, the original aim of the reported studies was to examine the effect of maternal EtOH consumption during pregnancy on the lipoprotein (Lp) profiles of the adult offspring measured by means of nuclear magnetic resonance spectroscopy. However, initial data suggested that compared to a maternal chow diet, the basal maternal liquid diet (without EtOH) had a significant effect on specific serum Lp of the adult offspring. The adult offspring of mothers who had consumed a basal liquid diet without EtOH exhibited significant increases in their plasma triglycerides (TG) and cholesterol content compared to adult offspring whose mothers consumed a chow diet. Further, there were significant increases in the offspring's VLDL and low density Lp (LDL) subfractions' particle number, regardless of whether the maternal liquid diet was ad libitum–fed, pair-fed, or EtOH-containing. The increase in offspring plasma TG was due to increases in specific VLDL subfraction particle numbers and not to increased TG content per particle. Similarly, the increase in plasma cholesterol was the result of elevated level of the very small LDL particles but not to an increased amount of cholesterol per LDL particle. These findings should be further examined in light of the widespread use of liquid diets in research to administer EtOH, especially for studies of fetal alcohol syndrome.

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Various experimental protocols have been used to administer ethanol (EtOH) to animals. During studies of the effects of alcohol on the liver, Lieber's laboratory (1–3) administered EtOH to rats by modification of a liquid diet used for the study of amino acid deficiencies as described by Forbes and Vaughan (4). These studies provided the initial approach to a solution to the problem of isocaloric nutrition of control animals. Over the next several years, Lieber and DeCarli elaborated the widespread usage of liquid diet formulations. This technique is now widely employed in alcohol research, and although other drinking paradigms are still used, the administration of EtOH via a liquid diet has become the gold standard, especially for fetal alcohol syndrome (FAS) research.

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Abbreviations: EtOH, ethanol; FAS, fetal alcohol syndrome; IDL, intermediate density lipoproteins; Lp, lipoproteins.

Ongoing studies in our laboratory examining the molecular causes of maternal diet–induced metabolic changes in the offspring (5–9) led to an unexpected finding as to the effect of a maternal liquid diet on the plasma lipoproteins (Lp) of the adult offspring as determined by NMR spectroscopy. Compared to the progeny of chow-fed mothers, adult rat offspring whose mothers consumed a commercial liquid diet based upon a formulation developed by Lieber and associates (but without EtOH), exhibited prominent increases in their circulating levels of VLDL triglycerides plus increased LDL-associated cholesterol. The offsprings' response to maternal liquid diet consumption varied among the subfractions in the various Lp components and, for some parameters, was sex-dependent.

EXPERIMENTAL PROCEDURES

Rat model. Virgin, Harlan-derived Sprague Dawley female rats 150–175 g (Harlan, Indianapolis, IN) ($n = 84$) were maintained on a 12-h day/12-h night cycle. The animals were fed commercial lab chow with a balanced liquid diet (Dyets, Inc., Bethlehem, PA) being available either *ad libitum* for 5 d prior to mating or at the start of pregnancy (sperm plug = day 0). The liquid diet was based upon the Lieber and DeCarli formulation (10) for dosing rats with EtOH. Each animal was randomly assigned to one of seven groups: Group 1 had EtOH added (36% of calories) to the liquid diet with 12% fat content (EtOH-fed, low fat), and Group 2 had EtOH added (36% of calories) to the liquid diet with 35% fat content (EtOH-fed, high fat). Group 3 was pair-fed an amount of the liquid diet with 12% fat isocaloric with that consumed by Group 1 in the previous 24 h, and Group 4 was pair-fed an amount of the liquid diet with 35% fat isocaloric with that consumed by Group 2 in the previous 24 h. Group 5 was *ad libitum*–fed the liquid diet with 12% fat without EtOH, and Group 6 was *ad libitum*–fed the liquid diet with 35% fat without EtOH. Group 7 females were fed *ad libitum* rat chow and tap water throughout their pregnancies. The surrogate group of pregnant animals was also fed *ad libitum* lab chow and tap water (see Fig. 1). The compositions of the liquid and chow diets are given in Table 1. All of the liquid diets contained comparable amounts of protein because of the known effect of maternal dietary protein levels on fetal development (11–14).

The pregnancies were allowed to go to term with the mothers being returned to commercial rat chow on day 20 of their

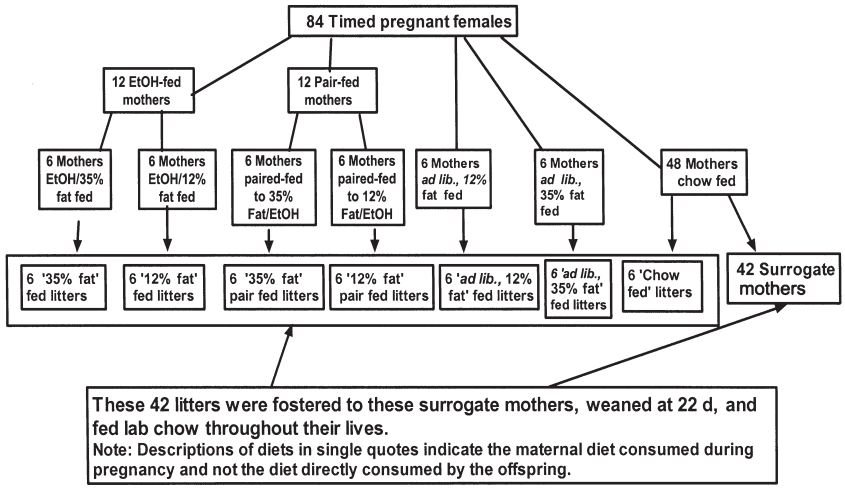

FIG 1. Diagram of the breeding paradigm used to generate the offspring utilized in the studies. Numbers in boxes indicate the minimum number of pregnant animals utilized. EtOH, ethanol.

pregnancy such that each female received the experimental or control diet for 20 d. At or before 2 d of age, the neonates were culled to four males and four females per litter, whenever possible, and surrogate-fostered to chow-fed mothers who had delivered within the previous 48 h. The surrogate-fostered pups were weaned at 22 d of age and fed commercial rat chow *ad libitum*. All pregnant animals were handled regularly to minimize stress, and they were weighed at 3-d intervals to maintain the isocaloric liquid diet pair feedings. The total experimental paradigm was run twice.

The East Carolina University Animal Care and Use Committee approved all animal procedures. The East Carolina University animal research facilities and program are accredited by the American Association for Accreditation of Laboratory Animal Care.

NMR studies. For the NMR studies, whole blood (0.50 mL) was collected via a tail vein from male and female offspring in 0.65-mL tubes containing EDTA (0.5 mg/tube). The animals were fasted for 8 h prior to the blood sample being collected to minimize circulating chylomicron levels. The samples were spun at room temperature at 10,000 rpm for 20 s using a microcentrifuge, and the resulting plasma super-

TABLE 1
Composition of Diets

	Liquid Diet Components from Dyets, Inc.				Chow Diet Testdiets, Inc.
Ingredient	12% Fat, Control Diet (g/L)	12% Fat, EtOH Diet (g/L)	35% Fat Control Diet (g/L)	35% Fat EtOH Diet (g/L)	Chow Diet (% of total)
Casein	41.4	41.4	41.4	41.4	0
L-Cystine	0.5	0.5	0.5	0.5	0.3
DL-Methionine	0.3	0.3	0.3	0.3	0.48
Corn Oil	2.5	2.5	8.5	8.5	6.4 (total from
Olive Oil	8.4	8.4	28.4	28.4	corn, soybean,
Safflower Oil	2.7	2.7	2.7	2.7	and animal)[a]
Maltose Dextrin	173.2	83.6	115.2	25.6	30.4 (starch)
Cellulose	10.0	10.0	10.0	10.0	1.0 (sucrose)
Salt Mix #210011	8.75	8.75	8.75	8.75	*
Vit. Mix #310011	2.5	2.5	2.5	2.5	*
Choline Bitartrate	0.53	0.53	0.53	0.53	1600 ppm as Cl
Xanthan Gum	3.0	3.0	3.0	3.0	0
95% EtOH	0	67.3 mL	0	67.3 mL	0
Water	q.s. to 1 L	q.s. to 1 L	q.s. to 1 L	q.s. to 1 L	10
	This diet contains 1.0 Kcal/mL, of which 18.0% are from protein, 12.0% are from fat, and 70.0% are carbohydrate.	This diet contains 1.0 Kcal/mL, of which 18.0% are from protein, 12.0% are from fat, 34.0% are from carbohydrate, and 36.0% are from ethanol.	This diet contains 1.0 Kcal/mL, of which 18% are from protein, 35% are from fat, and 47% are from carbohydrate.	This diet contains 1.0 Kcal/mL, of which 18% are from protein, 35% are from fat, 11% are from carbohydrate, and 36% are from ethanol.	This diet contains 4.1 Kcal/g, of which 26% are from protein, 14% are from fat, and 60% are from carbohydrate.

[a]Individual components of these ingredients can be found at http://www.labdiet.com/indexlabdiethome.htm. EtOH, ethanol.

natants (0.25 mL) were removed to 0.65-mL centrifuge tubes and immediately frozen in liquid nitrogen. The samples were transported on solid CO_2 to the laboratory of Dr. James Otvos (LipoScience, Raleigh, NC) for assay using the commercial LipoProfile NMR-based assay. This proprietary assay uses whole plasma in a single run at 46°C utilizing a 360 MHz NMR spectrometer.

Free FA assay. Nonesterified FA in plasma were analyzed by use of an assay kit (#990-75401) purchased from Waco Chemicals (Richmond, VA).

Statistical analyses of data. All assays were run in at least duplicate, and the results averaged to determine mean values. For all experiments, the value of *n* for each analysis was ≥ 6, including one animal of each sex, from each of six litters of a given maternal dietary treatment. Group means and standard errors, as well as *post hoc* testing of significant differences between means of the treatment groups, were calculated using the general linear model procedure of the SAS/PC statistical program (SAS Inc., Cary, NC). Statistically significant differences between group means was determined using a three-way ANOVA (maternal diet × offspring sex × offspring age) with $P < 0.05$ accepted as significant. Significant differences between individual groups were determined by the use of the least squared means test.

RESULTS

Compared to the offspring of mothers who consumed a chow diet during pregnancy, the offspring of liquid diet–fed mothers had increased amounts of the primary lipids associated with the VLDL, intermediate density Lp (IDL), and LDL fractions (Fig. 2). The low level of TG associated with the chylomicron fraction in both groups reflects the fact that the animals were food-deprived overnight before samples were taken. There were no significant differences in the results as a function of offspring age (data not shown). Initiating the liquid diet consumption 5 d prior to or at the start of the pregnancy had no significant effect on maternal consumption of the diet or on the offspring's outcome (data not shown). Varying the fat content of the maternal liquid diet did not appear to significantly alter the outcome (Fig. 3).

There were significant increases in the overall numbers of circulating VLDL, IDL, and LDL particles in the plasma of offspring of mothers who consumed the liquid diet (Fig. 4). But, other than an increase in the HDL fraction (Fig. 5), no significant differences were seen in the particle size of the Lp fractions. The increases in offspring Lp particle numbers occurred regardless of whether the maternal liquid diet was *ad libitum*–fed, pair-fed, or EtOH-containing. For example, offspring serum LDL particle content increased regardless of the maternal liquid diet composition or amount fed (Fig. 5).

The liquid diet altered the TG content of the individual VLDL subgroups in a distinct manner (Fig. 6A). Plasma VLDL subfractions 6, 4, 3, and 1 from the offspring of liquid diet–fed mothers had significantly higher TAG levels. VLDL subfraction particle concentration differences, shown in Figure 6B, illustrate that the increase in the number of TG-containing Lp particles was not uniformly distributed across the VLDL subfractions. Although there appeared to be little difference in the numbers of chylomicron particles (due to the animals being starved prior to blood being drawn), maternal consumption of the liquid diet significantly increased the number of circulating particles in the offspring's VLDL subgroups ($VLDL_6$, $VLDL_4$, $VLDL_3$, and $VLDL_1$). This finding is in contrast to the effect of maternal EtOH consumption, in which the increase in VLDL particle numbers and TG content was in the $VLDL_6$ particle (data not shown).

Dividing the TG content of each VLDL fraction (Fig. 6A)

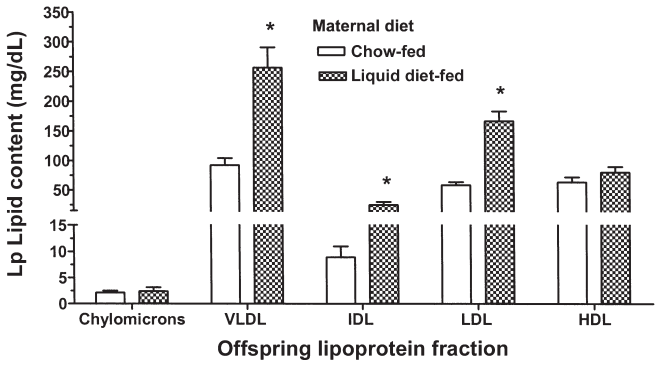

FIG 2. Combined data for adult male and female (90–150 d of age) offspring plasma TG associated with the chylomicron and VLDL lipoprotein (Lp) fractions as well as the cholesterol content of the intermediate density Lp (IDL), LDL, and HDL. In this and all other figures, the bars represent group mean values with SEM shown as a positive error bar. In this figure and all subsequent figures, an asterisk (*) indicates a group mean value for the offspring of liquid diet–fed mothers that is significantly different ($P < 0.05$) from that of the offspring of chow-fed mothers. TG were the primary lipid fraction associated with the chylomicron and VLDL fractions; cholesterol was the major lipid associated with all other Lp fractions.

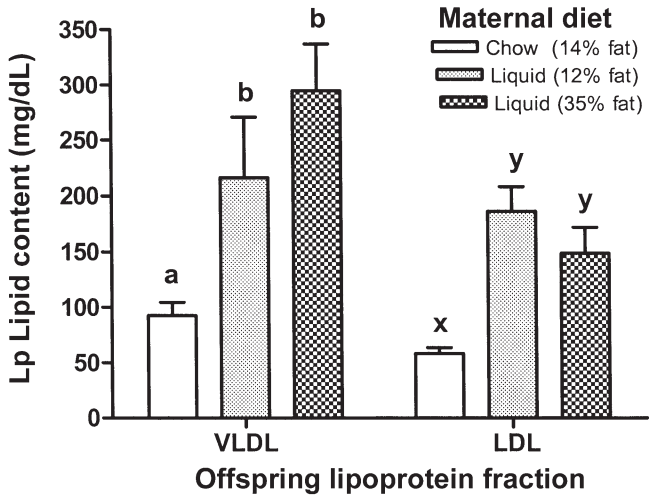

FIG 3. Comparison of the plasma VLDL TG content and LDL cholesterol content for offspring of chow-fed mothers with those of offspring from mothers consuming the liquid diet containing either 12% or 35% of calories as fat. For each Lp fraction, values for the maternal diets with a different letter are significantly different ($P < 0.01$). For abbreviation see Figure 2.

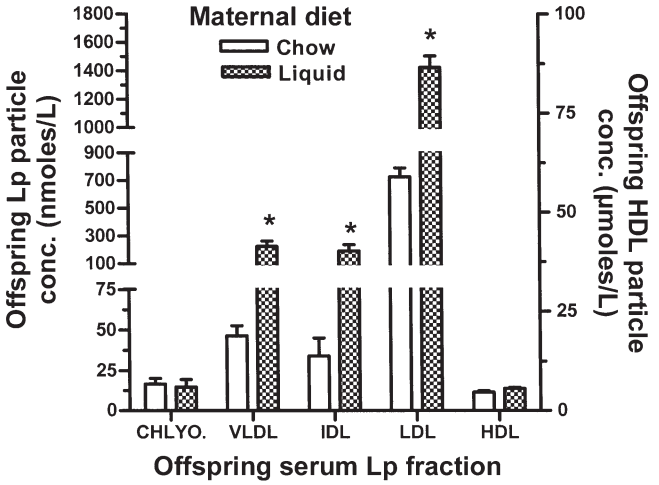

FIG 4. Effect of a maternal liquid diet on the plasma Lp particle concentrations of the offspring. The maternal liquid diet resulted in significant increases in the number of circulating VLDL, IDL, and LDL particles but no changes in the number of chylomicrons or HDL particles. Note the difference in the units for the right-hand y-axis (number of HDL particles). For abbreviations see Figure 2.

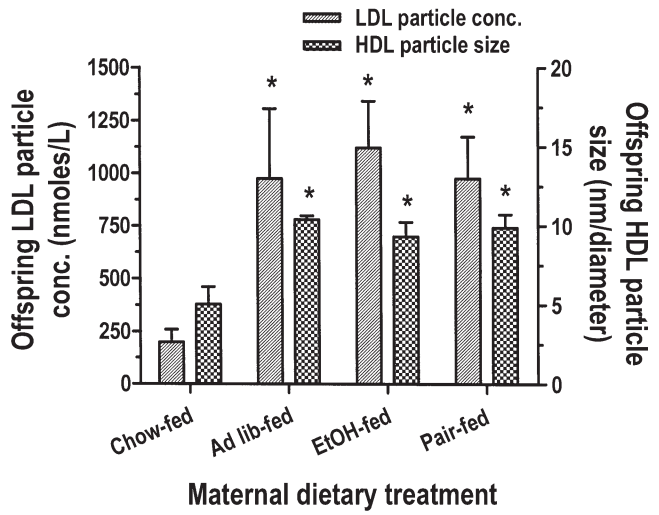

FIG 5. Comparison of serum LDL particle concentrations (left-hand axis) and HDL particle sizes (right-hand axis) from the offspring of chow-fed mothers to those for offspring of mothers consuming the liquid diet either *ad libitum* with EtOH, or pair-fed to the EtOH-fed mothers. The asterisk (*) indicates mean values that are significantly different from the same parameter in the progeny of chow-fed mothers. For abbreviation see Figure 1.

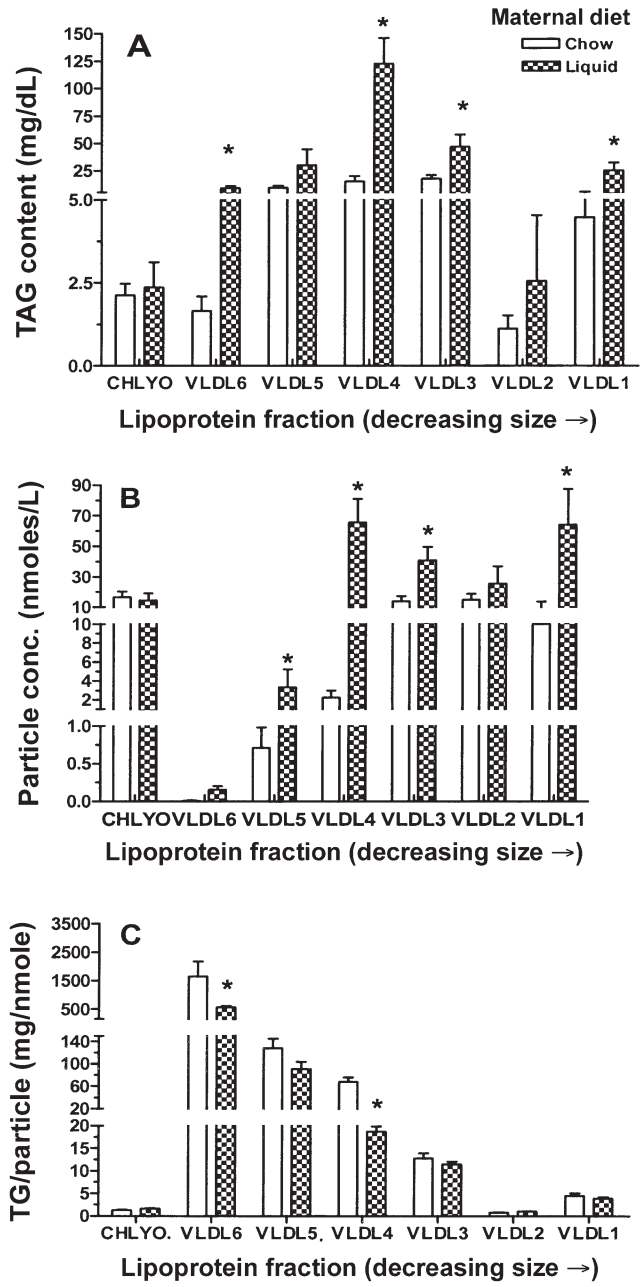

FIG 6. Comparison of (A) Lp particle concentration and (B) particle TG content for the chylomicron and individual VLDL subfractions, as well as (C) the ratio of TG/particle for each fraction for the offspring of chow-fed and liquid diet–fed mothers. For abbreviation see Figure 2.

by the number of particles in that VLDL fraction (Fig. 6B) illustrated that the TG concentration per Lp particle was either comparable or lower in all fractions for the offspring of liquid diet–fed mothers (Fig. 6C). Thus, the increase in plasma VLDL TG content for the offspring of liquid diet–fed mothers was the result of the large rise in the number of VLDL particles and not the result of an increased amount per VLDL particle.

Similar results were seen for the comparison of the offsprings' LDL particle numbers and plasma cholesterol concentration, except here the increases were associated with the smallest LDL particle (Fig. 7).

Sex-specific differences in the Lp subfraction distribution of both the TG- and cholesterol-rich Lp were also found. For example, male and female offspring whose mothers had consumed the liquid diet had different responses with respect to the amount of TG associated with the $VLDL_4$ subfraction (Fig. 8). We have previously reported what appeared to be sex-specific, maternal EtOH-induced changes in the TG content of the offspring's VLDL fraction (15).

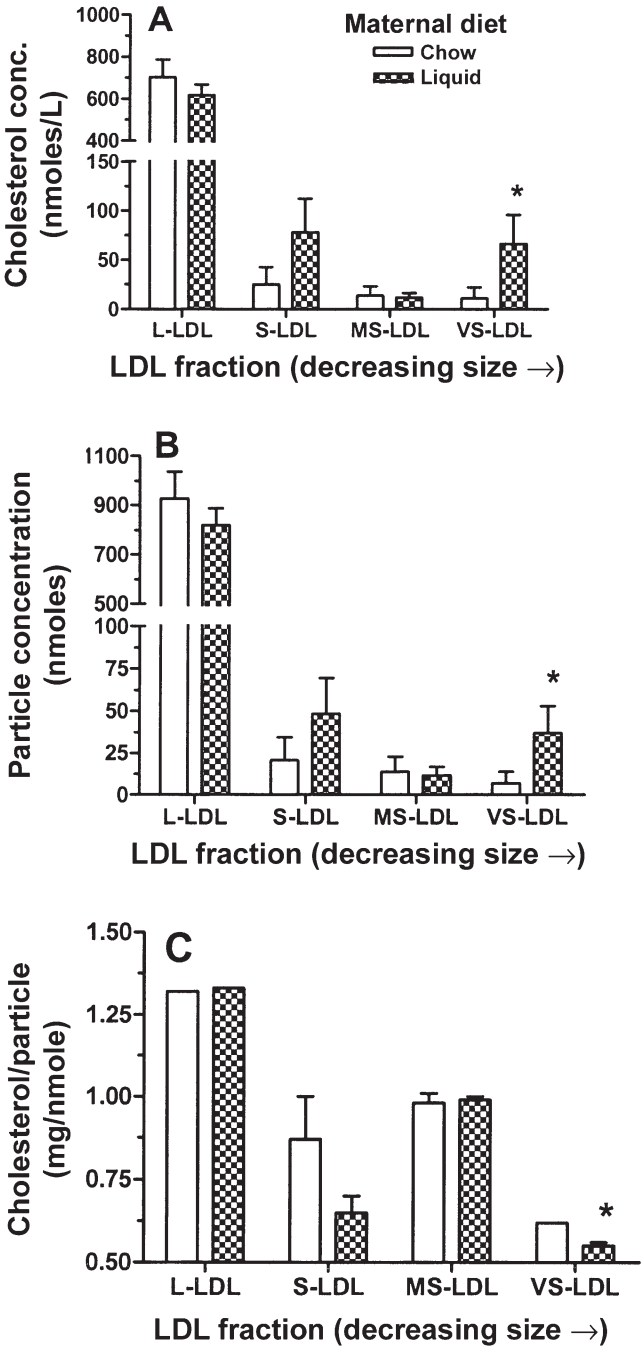

FIG 7. Comparisons of (A) LDL particle concentration and (B) particle cholesterol content for the individual LDL fractions, as well as (C) the ratio of cholesterol/particle for each LDL fraction for the offspring of chow-fed and liquid diet–fed mothers. L-LDL = large LDL, S-LDL = small LDL, MS-LDL = medium small LDL, and VS-LDL = very small LDL.

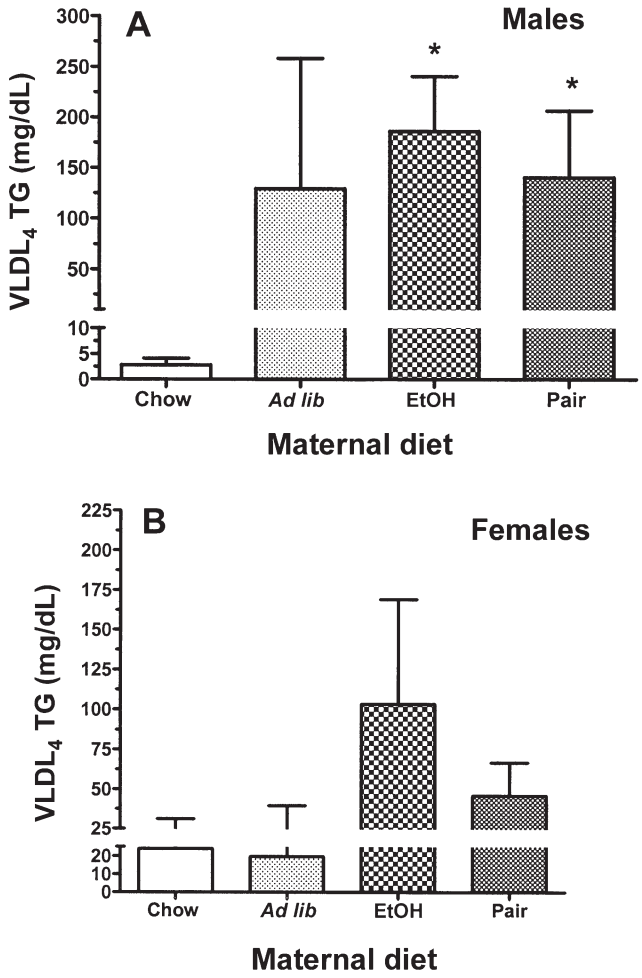

FIG 8. An example of a sex-specific difference in the offspring's Lp associated with the maternal liquid diet. (A) There were significant increases in the amount of TG associated with $VLDL_4$ for the male offspring of mothers who consumed the liquid diet. (B) The female offspring showed no significant differences as a function of maternal diet except that the female offspring of mothers who consumed EtOH in the liquid diet showed a tendency to have increased TG. For abbreviations see Figures 1 and 2.

DISCUSSION

The observations reported here suggest that *in utero* exposure to a maternal liquid diet based upon the Lieber formulation can significantly alter the adult offspring's Lp. Studies to examine the effects of maternal EtOH on the adult offspring's insulin sensitivity showed significant differences in the plasma Lp of the offspring whose mothers consumed the liquid diet, even without EtOH. The Lieber diet formulation has been used in hundreds of studies, and although other drinking paradigms are still used, the administration of EtOH via the liquid diet formulation has become the gold standard, especially for FAS research.

It is known that the source and amount of maternal dietary calories consumed during pregnancy can affect the offspring's development and growth (12,16–18). Further, intake of individual proteins from different dietary sources has been shown to alter Lp particle composition and metabolism (19–22). Also, simple dietary sugars, but not starch, have been shown to modulate the response to protein (23).

The current results suggest that maternal consumption during pregnancy of a liquid dietary formulation widely used in the study of the fetal effects of EtOH can cause significant,

long-term changes in the offspring's plasma Lp. Not only did the maternal liquid diet appear to be associated with increases in the offspring's TG and cholesterol levels but the increases appeared to result from changes in specific lipoprotein subfractions. Based on current research (24–27), such maternal diet–induced differences could reflect important changes in the offspring's Lp metabolism.

Evaluation of the increased lipid content of individual Lp subfractions and the changes in the particle numbers of those subfractions suggests that the increase in offspring plasma lipid levels were the result of increases in specific Lp subfraction particle numbers and not of increases in the amount of lipid per particle. Thus, the increase in offspring plasma TG associated with maternal consumption of the liquid diet was the result of a significant increase in the number of VLDL particles, and not the result of an increase in the amount of TG per particle.

The differences observed in the $VLDL_4$ subfraction may result from sex-related differences in the offspring's response to the maternal diet for this fraction (see Fig. 8). There are known sex-related differences in Lp profiles (28,29), and similar differences were seen in the current studies. That is, in general, females offspring had lower levels of Lp (data not shown). Further, certain of the changes observed here were sex-specific, specifically with regard to which Lp subfraction was involved, but the magnitude of the overall increases in TG and cholesterol was significant for both male and female offspring.

For LDL, the increase in offspring cholesterol associated with maternal consumption of the liquid diet was also the result of a significant increase in the number of very small LDL particles and not due to an increase in the amount of cholesterol per particle, the value of which actually decreased in the offspring of liquid diet–fed mothers. An increase in the number of the more athrogenic very small LDL particles would suggest that these offspring may be at increased risk for cardiovascular disease.

The development of a rapid, single-scan NMR method (30–34) has proven to be a powerful tool for the study of Lp and their role in the pathophysiology of cardiovascular disease. The ability to simultaneously determine plasma Lp particle numbers, particle size distribution, and the concentration of the major lipid component associated with each particle size range is an invaluable tool. It is well-recognized that catabolism within an Lp class, such as removal of TG from large VLDL particles by lipoprotein lipase, results in smaller particles that can differ in their metabolic rates and athrogenicity. Also, the exchange of lipid moieties and proteins between Lp may occur, leading to changes in Lp metabolism. Thus, unless one is able to determine the types and amounts of Lp in circulation at a given time, it is difficult to deduce what changes have occurred as the result of an experimental treatment. Because of this metabolic interaction/exchange between Lp, the ability of the NMR method to determine multiple parameters for all major Lp in a single sample is extremely valuable.

The variation in the distribution of TG across the VLDL subfractions observed here demonstrates the significant advantage of using NMR spectrometry to investigate plasma Lp. Only NMR analyses provide such detailed data without extensive sample preparation, including difficult fractionation of the plasma Lp, and even then, with other methods there is a high likelihood that much detail about lipid distribution would be lost.

Although nutritional or chemical differences between the maternal liquid diets and the chow diet are potential mechanisms that could be responsible for the differences observed in the offspring's Lp, such differences are not immediately apparent in the diet formulations (35) and, in fact, significant research (cited earlier) has been carried out to minimize differences between the Lieber-DeCarli formulation and chow diets designed for reproduction and growth. Thus, if the reported increases in the offspring's Lp and associated lipid levels are proven accurate, the results would raise significant questions about the role of the Lieber-DeCarli diet in alcohol research and especially in such studies related to fetal plasma lipids. Whatever their cause, the magnitude of the observed differences suggests that such changes could have significant metabolic consequences for the offspring.

ACKNOWLEDGMENTS

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Effect of Low Vitamin A Status on Fat Deposition and Fatty Acid Desaturation in Beef Cattle

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ABSTRACT: A group of Angus beef cattle was removed from temperate pastures and fed a very low β-carotene cereal-based ration in a feedlot for over 300 d. Half the group was supplemented weekly with retinyl palmitate (at the rate of 60,000 IU vitamin A/100 live weight (LW)/day), sufficient to offset clinical vitamin A deficiency; the other half received no supplement. Blood was sampled from all animals at biweekly intervals to assess β-carotene and vitamin A status. Adipose tissue was sampled by biopsy on three occasions throughout the experimental period and at slaughter to assess FA composition. Muscle was sampled at slaughter to determine the intramuscular fat content. The mean plasma concentration of β-carotene of all animals fell from an initial value of 20.1 to 5.2 μg/mL at 14 d, to 1.4 μg/mL at 35 d, and to zero at 105 d. Mean vitamin A in plasma was not significantly different between the treatment groups initially. The values then rose to almost twice their initial values by 35 d, but subsequently fell to below initial values by day 119. Thereafter, plasma vitamin A of the supplemented group was significantly greater than that of the unsupplemented group ($P < 0.05$). Muscle samples at slaughter from supplemented animals contained significantly ($P < 0.01$) more intramuscular lipid (13.0 vs. 9.6%). Major changes occurred over time in FA composition in both groups. Saturated FA decreased as monounsaturated FA increased over the first 60 d. An index of desaturation of FA was significantly lower ($P < 0.001$) in the vitamin A-supplemented group than in the nonsupplemented group. M.P. of the adipose tissue of nonsupplemented animals was 32.3°C, significantly less ($P < 0.05$) than that of supplemented animals (34.1°C). Feeding vitamin A was associated with less intramuscular fat but with a less desirable (less unsaturated, more solid) FA profile.

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Intramuscular fat (IMF) deposition and the desaturation of fat have a major influence on the quality of beef. The expression of both traits is partly influenced by the genes controlling adipocyte differentiation (1) and by stearoyl-CoA desaturase (SCD) activity (2), but both traits are also subject to environmental (chiefly, dietary) factors. Cattle reared on pastures do not grow to maturity or fatten as rapidly as those reared on high-energy cereal-based diets, and they have higher levels of saturated FA (SFA) (3). Differences both in fattening and in the degree of saturation between pasture-fed and cereal-fed animals can be partly attributed to either inhibition of adipocyte differentiation or SCD gene expression by vitamin A derived from dietary β-carotene (2).

Japanese cattle are chiefly cereal-fed, reared on low β-carotene and vitamin A diets to produce animals with high marbling (i.e., IMF). Studies of cattle under these conditions have found that marbling scores are negatively related to blood vitamin A (4,5). Although historically Australian beef cattle have been reared on pastures and thus consume varying quantities of provitamin A (β-carotene), it is now relatively common in Australia (near 30% in 2003) to fatten animals with low-carotene cereal rations (6). The residual effects of prior feeding on pastures, however, may well affect the vitamin A status of cereal-fed animals for some time or alternatively, lack of vitamin A may lead to deficiency. The present experiment was designed to determine the effect of rations low in provitamin A and vitamin A on IMF deposition and FA desaturation in cattle after rearing on pasture.

MATERIALS AND METHODS

Animals and management. Twenty, 12-mon-old Angus steers, all progeny of one sire with a high propensity for marbling, were used in the study. The steers were removed from pasture and placed in feedlot facilities of the Department of Primary Industries, Rutherglen, Victoria, for 10 mon. In the feedlot, they were fed standard commercial feedlot rations without vitamin A, composed of triticale and wheat as the grain source (95 and 5%, respectively), canola meal as the protein source, and triticale straw as the fiber source. A premix containing all essential vitamins and minerals, except vitamin A, was added to the ration. The steers were fed three rations: a 12.8% protein ration for the first 100 d, a 7.5% protein ration for the next 100 d, and finally a 2.0% protein ration for the last 100 d. Similar to values from other published sources (7), laboratory analysis showed that the β-carotene content of the feed rations did not exceed 5 mg/kg dry matter. Prior to grain feeding, the steers were allocated to two treatment groups: vitamin A nonsupplemented (A−) and vitamin A supplemented (A+), with each

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Abbreviations: IMF, intramuscular fat; MUFA, monounsaturated FA; SCD, stearoyl-CoA desaturase; SFA, saturated FA.

group comprising 10 animals. The allocation to treatment groups was based on feedlot entry weight; animals were ranked by live weight (LW) and then allocated alternately to either group. During the feeding period, blood was sampled biweekly for plasma vitamin A (retinol) and β-carotene concentration. Adipose tissue was sampled by biopsy on three occasions. Ultrasound scans every 50 d provided indirect measures of IMF. All the steers were kept in one pen, and once a week the (A+) group was taken to an adjacent pen and briefly fed feed supplemented with retinyl palmitate at the rate of 60,000 IU vitamin A/100 kg LW/d. At the start of the trial, the mean weight (± SEM) of all cattle was 354.9 ± 6.1 kg. The Animal Ethics Committee of the Department of Primary Industries, Rutherglen, approved of all measurements and sampling of tissues from cattle (application number 200104).

Biopsy sampling. Fat samples were removed by biopsy conducted at day 0, day 150, and day 268 of the experiment by methods described elsewhere (8). In brief, a 3–5 cm incision was made at a site at the base of the tail of a restrained animal that had received local anesthetic at the marked site. Three to five gram of fat was removed using a scalpel and the wound was treated with antibiotic powder before closure with surgical metal staples. The animals were injected with Terramycin® (Pfizer, Australia), and the sampling site was protected against dust and flies with Stockholm Tar® (Joseph Lyddy, Waproo Pty. Ltd., Canterbury, Victoria, Australia). Fat samples were placed in a sieve, washed with deionized water, then placed in vials prior to freezing in liquid nitrogen for storage at −80°C.

Ultrasound scanning procedures. Intramuscular fat prior to slaughter was assessed by ultrasound scanning of musculus longissimus dorsi between the 12th and 13th rib position of animals in both treatment groups. A certified scanner using an Aloka 500V with a 3.5-MHz, 17.2-cm linear array transducer measured IMF in individual animals every 50 d (9).

Vitamin A and β-carotene determination. Vitamin A (retinol) and β-carotene levels in blood were determined as described elsewhere (10,11). In brief, the proteins in the blood plasma (500 μL) were precipitated with 500 μL of ethanol. α-Tocopherol acetate was added as an internal standard. After vortexing, retinol and β-carotene were extracted with 600 μL of hexane, samples were centrifuged at 10,000 × *g* for 3 min, and 400 μL of hexane extract was dried under a stream of nitrogen. The residue was resuspended in a mobile phase of methanol/hexane/water (90:8:2, by vol) and run on a high-performance liquid chromatograph (model 1100; Hewlett-Packard, Palo Alto, CA). Separation was achieved using a Spherisorb ODS column (5 μm, C18 250 × 4.6 mm), protected by a precolumn guard cartridge. The mobile phase was used at a flow rate of 2.0 mL/min. Analytical standards of β-carotene, retinal, and α-tocopherol acetate (internal standard) were obtained from Fluka (Buchs, Switzerland). The β-carotene content of the various ingredients of feed rations was determined by methods published elsewhere (12).

IMF content, FA composition, and m.p. Muscle samples (musculus longissimus dorsi) were trimmed of all visible fat, and a subsample (~100 g) was homogenized in a food processor. Fat content was extracted from an accurately weighed subsample (~2 g) of the homogenized muscle using chloroform/methanol (2:1, vol/vol) as the solvent (13,14). FA composition of adipose tissue lipids was determined by GC. Methyl esters of FA were prepared under acid-catalyzed conditions from subsamples of adipose tissue (~20 mg) as described elsewhere (4). Individual FA were identified and quantified with use of a Hewlett-Packard gas chromatograph (5890A II) fitted with a capillary column (BPX70; SGE, Melbourne, Australia). The system allowed for the identification of *cis/trans* isomers. Unsaturated FA reported herein were identified relative to the carboxyl end (Δ convention). Total SFA and total monounsaturated FA (MUFA) were expressed as the sum of the individual FA (ΣSFA and ΣMUFA). A desaturation index was calculated from the sum of the saturated species (ΣSFA) and from the sum of the *cis* monounsaturated species (Σ*cis* MUFA). The index was calculated as ΣSFA × 100/(ΣSFA + Σ*cis* MUFA). At the time of subsampling adipose tissue for FA analysis, similar subsamples were used to determine m.p. as described elsewhere (13).

Slaughter measurements. The animals were transported approximately 1000 km to a meat works at Murray Bridge, South Australia, 2 d before the slaughter. After arrival at the meat works, the steers had *ad libitum* access to water and hay. Liver samples were collected on the slaughter floor and muscle samples in the boning room on the following day.

Statistical analyses. Least squares ANOVA for the entire data set was initially carried out. A model including LW and plasma vitamin A at day 0 as covariates and treatment (supplemented vs. unsupplemented vitamin A) was initially fitted to the data. Since these covariates were not significant for any of the traits measured, a formal model was deemed unnecessary and a simple *t*-test was used to compare treatment means.

RESULTS

Plasma β-carotene and vitamin A (retinol) levels altered considerably when cattle were removed from pasture and fed a cereal diet in the feedlot (Fig. 1). Plasma β-carotene (mean of all animals) was 20.1 μg/mL when the animals were removed from pasture and entered the feedlot. This value fell abruptly to 5.2 μg/mL by day 14, when half the animals began supplementation with vitamin A, and continued to decrease. The value was still positive at day 77, but fell to zero by day 90 and thereafter (Fig. 1). Plasma retinol of both groups increased considerably with the low-carotenoid cereal diet by day 35, then fell over the next two measurements, before increasing again at day 77. Both groups then decreased to lower than initial values by day 90, when β-carotene reached zero. Despite similar responses by both groups, the supplemented group demonstrated a significantly higher level of plasma retinol ($P < 0.05$) than the unsupplemented group at day 105 and remained significantly higher for the remainder of the experiment. Thus, there was no significant difference between the plasma retinol concentrations of both groups at day 0, but there was a highly significant difference at day 294.

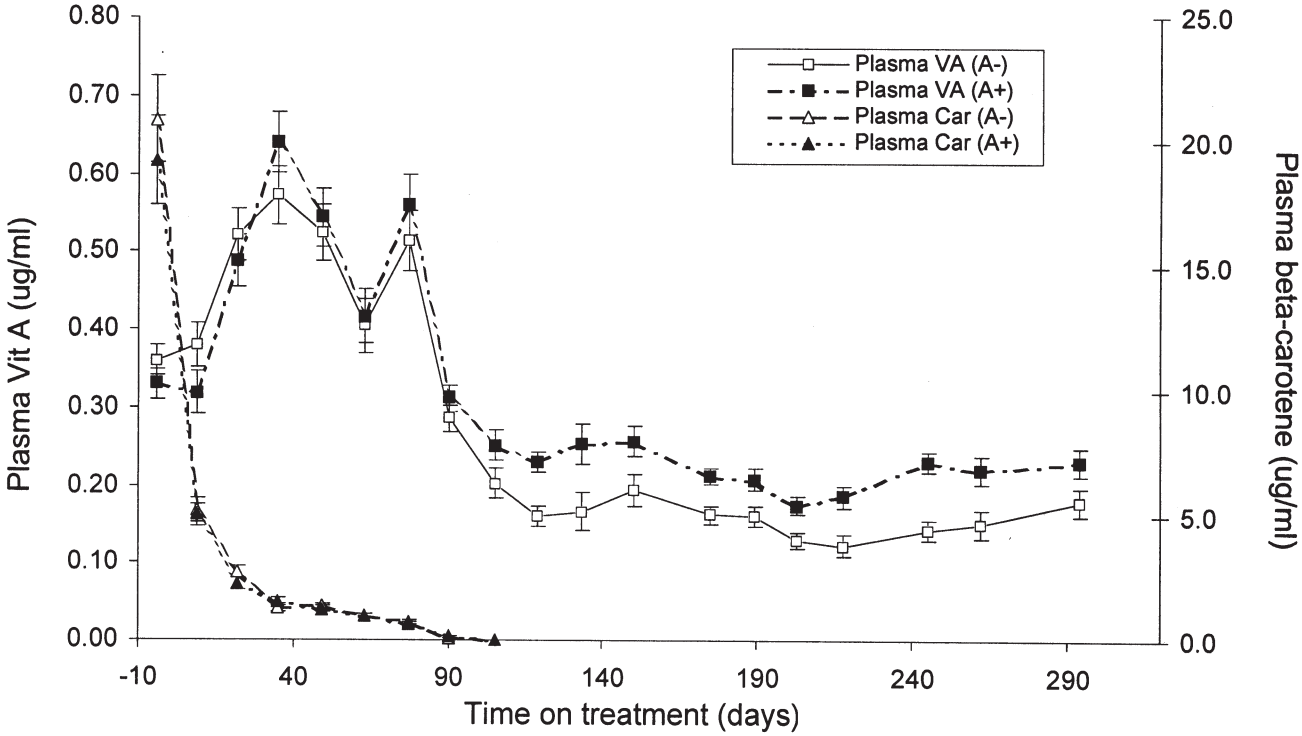

FIG. 1. Plasma vitamin A (retinol) and plasma β-carotene after pasture-fed cattle were transferred to a carotene-free cereal diet in the feedlot, half of which were supplemented with dietary vitamin A (A+); the remainder were unsupplemented (A–). Plasma vitamin A was significantly greater ($P < 0.05$) in A+ animals than in A– animals after day 105.

The IMF content as determined by ultrasound scanning of the two groups of cattle prior to cereal feeding was 3.2 and 3.0% for the unsupplemented and supplemented groups, respectively (Table 1). The mean (± SE) IMF content of tissue collected at slaughter as measured by solvent extraction was 9.6 ± 0.8% (wet weight) in animals from the supplemented group and 13.0 ± 0.6% (wet weight) in animals from the unsupplemented group. The effect of supplementation of cattle with vitamin A was associated with a 26% reduction in IMF concentration. The difference between the values was highly significantly ($P = 0.0025$). LW, LW gain, and rib fat depth showed no difference between treatments.

Both treatment groups, A– and A+, became less saturated and more monounsaturated after cereal feeding commenced (Table 2). The major contributor to this decrease in SFA was the decrease in stearic acid (18:0), which fell by 8%. There was little change in the 14:0 and 16:0 levels. Associated with this decrease in stearic acid was an increase in the content of oleic acid (18:1 *c*9), the product of C18 desaturation. Most of these changes occurred by day 150, although there was some further change by day 270. These changes in FA species also altered the m.p., causing it to decrease by at least 9°C between day 0 and day 270.

TABLE 1
Mean Values of Intramuscular Fat Content (%) from Unsupplemented (A–) and Supplemented (A+) Cattle After Removal from Pasture (day 0) and After Cereal Feeding in the Feedlot

Treatment Animals	A– ($n = 10$)	A+ ($n = 10$)	Significance
Intramuscular fat[a] (%)—day 0	3.2 ± 0.2[b]	3.0 ± 0.2	NS
Plasma vitamin A (μg/mL)—day 0	0.359 ± 0.020	0.330 ± 0.017	NS
Live weight (kg)—day 4	360.0 ± 7.1	349.8 ± 10.2	NS
Rib fat depth[a] (mm)—day 0	2.5 ± 0.3	2.6 ± 0.4	NS
Plasma vitamin A (μg/mL)—day 294	0.177 ± 0.014	0.229 ± 0.023	$P < 0.05$
Intramuscular fat[c] (%)—slaughter (day 307)	13.0 ± 0.6	9.6 ± 0.8	$P < 0.05$
Live weight (kg)—slaughter (day 307)	667.8 ± 8.2	691.8 ± 16.1	NS
Live weight gain (kg/d) (311 days)	1.02 ± 0.03	1.09 ± 0.05	NS
Rib fat (mm)—slaughter	20.3 ± 1.5	20.5 ± 1.1	NS

[a]Assessed by ultrasound scanning.
[b]Mean ± SE.
[c]Assessed by chemical extraction.

TABLE 2
Mean Values[a,b] of the FA Components (wt% total FA) of Subcutaneous Fat from Cattle Sampled After Removal from Pasture (day 0) and at 150 and 268 d of Cereal Feeding in the Feedlot

	Sampling at day 0		Sampling at day 150		Sampling at day 268	
Treatment	A–	A+	A–	A+	A–	A+
Animals	*n* = 10	*n* = 10	*n* = 10	*n* = 10	*n* = 10	*n* = 10
FA						
14:0	4.37 ± 0.19[a]	5.17 ± 0.19[a]	4.08 ± 0.19[b]	4.30 ± 0.19[b]	3.60 ± 0.19[c]	3.70 ± 0.19[d]
14:1 (*c*9)	0.53 ± 0.16[a]	0.89 ± 0.16[b]	1.68 ± 0.16[c]	2.03 ± 0.16[d]	1.64 ± 0.16[c]	1.70 ± 0.16[c]
15:0	0.64 ± 0.05[a]	0.72 ± 0.05[a]	0.66 ± 0.05[a]	0.72 ± 0.05[a]	0.57 ± 0.05[a]	0.63 ± 0.05[a]
16:0	26.83 ± 0.56[a]	28.04 ± 0.56[c]	28.87 ± 0.56[b]	28.20 ± 0.56[a]	26.27 ± 0.56[a]	27.07 ± 0.56[c]
16:1 (*c*7)	3.27 ± 0.34[a]	3.90 ± 0.34[a]	4.76 ± 0.34[b]	5.41 ± 0.34[b]	4.78 ± 0.34[b]	4.91 ± 0.34[b]
16:1 (*t*5)	0.76 ± 0.04[a]	0.85 ± 0.04[a]	0.59 ± 0.04[b]	0.67 ± 0.67[b]	0.60 ± 0.04[b]	0.62 ± 0.04[b]
17:0	1.26 ± 0.07[a]	1.26 ± 0.07[a]	1.43 ± 0.07[b]	1.55 ± 0.07[b]	1.37 ± 0.07[c]	1.45 ± 0.07[b]
18:0	18.94 ± 0.49[a]	17.41 ± 0.49[c]	10.96 ± 0.49[b]	10.17 ± 0.49[b]	10.00 ± 0.49[b]	10.36 ± 0.49[b]
18:1 (*t*11)	5.31 ± 0.41[a]	5.09 ± 0.41[a]	1.95 ± 0.41[b]	1.34 ± 0.41[b]	1.38 ± 0.41[b]	3.09 ± 0.41[c]
18:1 (*c*9)	32.53 ± 0.74[a]	31.50 ± 0.74[a]	41.38 ± 0.74[b]	41.51 ± 0.74[b]	45.32 ± 0.74[c]	43.07 ± 0.74[c]
18:1 (*c*11)	0.71 ± 0.50[a]	0.60 ± 0.50[a]	1.90 ± 0.50[a]	2.21 ± 0.50[b]	2.61 ± 0.50[b]	1.89 ± 0.50[b]
18:2 (*c*9,*c*12)	1.36 ± 0.09[a]	1.24 ± 0.09[a]	1.23 ± 0.09[a]	1.10 ± 0.09[b]	1.34 ± 0.09[a]	1.16 ± 0.09[b]
18:3 (*c*9,*c*12,*c*15)	0.82 ± 0.04[a]	0.82 ± 0.04[a]	0.08 ± 0.04[b]	0.24 ± 0.04[c]	0.16 ± 0.04[b]	0.10 ± 0.04[b]
18:2 (*c*9, *t*11)(CLA)	1.23 ± 0.06[a]	1.08 ± 0.06[a]	0.32 ± 0.06[b]	0.45 ± 0.06[b]	0.28 ± 0.06[b]	0.19 ± 0.06[c]
ΣSFA[c]	52.08 ± 0.79[a]	52.62 ± 0.79[a]	46.03 ± 0.79[b]	44.98 ± 0.79[b]	43.85 ± 0.79[c]	46.97 ± 0.79[b]
Σ*cis* MUFA[d]	43.15 ± 0.71[a]	42.87 ± 0.71[a]	52.31 ± 0.71[b]	53.18 ± 0.71[b]	54.37 ± 0.71[c]	51.56 ± 0.71[b]
Desat. index (%)	39.85 ± 0.88[a]	39.62 ± 0.81[a]	51.34 ± 0.67[b]	52.91 ± 0.80[b]	55.81 ± 0.97[c]	53.02 ± 0.61[b]
m.p. (°C)	43.0 ± 0.47[a]	43.05 ± 0.47[a]	37.9 ± 0.47[b]	37.2 ± 0.47[b]	32.3 ± 0.47[d]	34.1 ± 0.47[c]

[a]Mean ± SE.
[b]Values with different superscripts differ significantly at $P < 0.05$ at least.
[c]ΣSFA, total saturated FA.
[d]Σ*cis* MUFA, total *cis* monounsaturated FA.

Between treatments, total SFA in unsupplemented animals decreased from 52.08 to 43.85%, while supplemented animals decreased to 46.97%, a value significantly higher than that of the unsupplemented animals. Simultaneously, total *cis* MUFA of unsupplemented animals increased significantly to 54.37% from 43.15%, while supplemented animals only increased to 51.56%. CLA was significantly higher in the nonsupplemented animals after 270 d of cereal feeding. Supplementation with vitamin A lessened the degree of desaturation as assessed by an index that included the major SFA and MUFA. This was also demonstrated by the difference in m.p. between treatment groups. In the nonsupplemented animals, the average m.p. was 32.3°C, significantly lower than that of supplemented animals at 34.1°C ($P < 0.05$). The effect of vitamin A supplementation on the desaturation of SFA was exemplified in the difference of the degree of desaturation that occurred after 270 d of cereal feeding with and without vitamin A supplementation (Table 2). Desaturation indices rose from approximately 39% to in excess of 51%. At 268 d, unsupplemented animals had a value of 55.81%, significantly greater ($P < 0.05$) than that of supplemented animals at 53.02%.

Desaturation indices were inversely related ($P < 0.001$) to plasma vitamin A levels (Table 3) after 268 d of cereal feeding. Plasma vitamin A and the index of desaturation in unsupplemented animals measured 0.149 μg/mL and 55.81%, respectively, and in supplemented animals measured 0.220 μg/mL and 53.10%, respectively. The difference between treatments of the various parameters was significantly different in all cases ($P < 0.05$ or greater). The difference in vitamin A status between treatments at the end of cereal feeding was further demonstrated by slaughter liver vitamin A and β-carotene levels, which were significantly different between treatments ($P < 0.001$).

TABLE 3
Mean Values of Plasma Vitamin A and FA Desaturation Index of Unsupplemented (A–) and Supplemented (A+) Cattle After Removal from Pasture (day 0) and After 268 d of Cereal Feeding in the Feedlot[a]

Treatment	A–	A+	
Animals	*n* = 10	*n* = 10	Significance
Plasma vitamin A (μg/mL)—day 268	0.149 ± 0.023[b]	0.220 ± 0.011	$P < 0.05$
Desaturation index (% total FA)—day 268	55.81 ± 0.97	53.02 ± 0.61	$P < 0.001$
Liver retinol (μg/g)—at slaughter	12.4 ± 3.8	223.4 ± 26.0	$P < 0.001$
Liver β-carotene (μg/g)—at slaughter	0.04 ± 0.01	0.50 ± 0.10	$P < 0.001$

[a]Liver vitamin A and liver β-carotene at slaughter are also shown.
[b]Mean ± SE.

DISCUSSION

It has been recognized for some years that differentiation of animal fat cells is controlled by communication between individual cells or between cells and the extracellular environment (1). Most effects have been determined in cell lines and primary cultures, where various hormones, cytokines, and growth factors have been shown to have positive and/or negative effects on adipocyte differentiation. Retinoic acid, a particularly potent form of vitamin A, is one such agent; it has been shown to inhibit adipocyte differentiation of adipocyte cell lines and primary preadipocytes (15–17). Livestock animal studies in Japan (4) showed that in a small number of cattle (4 per treatment), animals injected with vitamin A showed visibly less muscular fat (marbling) than those that did not receive vitamin A. In the experiment, 15-mon-old steers with a starting serum vitamin A level of nearly 0.4 μg/mL were fed a vitamin A-free diet. Without supplementation, this value fell to approximately 0.07 μg/mL after 15 mon. The unsupplemented group had a significantly higher marbling score than the steers injected with 20 mL vitamin A palmitate (303 mg as vitamin A alcohol) every 2 mon, whose serum vitamin A was maintained above 0.3 μg/mL for most of the measurement period.

In the present experiment, plasma vitamin A levels were very similar to the Japanese study mentioned above (4), prior to a period of cereal feeding (0.33 and 0.36 μg/L), and IMF, determined from muscle samples removed at slaughter, was 35% higher in the nonsupplemented animals. Vitamin A levels rose soon after cereal feeding commenced to values in excess of 0.5 μg/mL before decreasing to below initial values by 90 d. This rise in plasma vitamin A in the absence of either vitamin A or β-carotene in the diet was maintained until plasma β-carotene could no longer be detected, indicating vitamin A was being formed by cleavage of β-carotene in the body stores by the enzyme 15,15′-dioxygenase. This enzyme normally acts in the small intestine with dietary carotene, but the enzyme is also present in the liver (18,19). In previous studies from this laboratory, increased IMF was observed in young sheep fed a cereal diet, similar to the cattle herein. Half the lambs were fed provitamin A (β-carotene) by inclusion of green feed in their diet (20). At slaughter, the lambs on the low provitamin A (41 mg/d) diet had a mean IMF level of 6.3%. The lambs on the high provitamin A (890 mg/d) diet had a mean level of 4.9%. This 29% increase in nonsupplemented animals was a significant response, not dissimilar to that in the cattle herein.

In the cattle of the present experiment and in the Japanese cattle referred to above, the supplements of vitamin A amounted to 60,000 and 35,000 IU/100 kg LW/d, respectively. These intakes of vitamin A are relatively small, however, compared with those of cattle grazing green pastures, which can range from 100,000 to 400,000 IU vitamin A/100 kg LW/d derived from β-carotene (200–800 mg β-carotene/kg dry matter). Thus, the effects of vitamin A in a grazing animal would be far greater than in animals fed cereal in a feedlot with or without supplementation (<5 mg β-carotene/kg dry matter). This is demonstrated in grass-fed cattle, even when selected for IMF, as they only achieve IMF levels of approximately 5.8% at a LW of 550 kg (21). This is comparable to the 13.0% IMF content of the unsupplemented cattle of similar breed and LW in the experiment reported here. In animals receiving a limited supplement of vitamin A herein, IMF was measured at 9.6%, a value that was anticipated based on an intermediate level of provitamin A (carotene) or vitamin A intake.

Coincident with the increase in IMF was an increase in ΣMUFA in adipose tissue. At the end of cereal feeding, there was also a significant increase in ΣMUFA when vitamin A was not supplemented in the diet compared with that of supplemented animals. FA desaturation of adipose tissue increased as animals moved from a high provitamin A pasture diet to a low vitamin A cereal diet. CLA, a FA synthesized in cattle by biohydrogenation in the rumen and in adipose tissue from *trans* vaccenic acid by the action of a desaturase enzyme (22), was also significantly higher in the nonsupplemented animals. The significantly higher value of desaturation in nonsupplemented animals was inversely associated with vitamin A status in terms of plasma and liver vitamin A levels, and also liver β-carotene. High IMF and high MUFA are both properties that attract premium prices for beef.

The propensity of herbivores to deposit lipid in muscle is dependent on an interaction between genetic factors displayed differently by the species or breed and environmental factors such as dietary β-carotene. Angus, Hereford, Wagyu, and Jersey breeds mature earlier than other European breeds or tropical cattle and have higher IMF levels when reared on cereal feeds in the feedlot (3,23). At pasture, however, the differences in IMF are small (3,21). Although on occasion this can be partly attributed to lesser energy intake and growth rate, the lower IMF content of pasture feeding compared with cereal feeding can also be attributed to β-carotene consumption. Lower fat deposition with carotene diets can be explained by the negative effect of retinoic acid, a potent form of vitamin A (1) that is capable of inhibiting differentiation of preadipocytes. Retinoic acid has also been shown to inhibit Δ^9-desaturase activity in rats (24) and to regulate SCD gene 1 expression (2). The enzyme is present in cattle adipose tissue (25) and is significantly higher in Jersey cattle that absorb more β-carotene than other breeds and form less retinoic acid (11). Genetic polymorphisms in the SCD gene have been associated with breed differences in FA profile within the Wagyu breed (26). Studies suggest SCD transcriptional activity may account for some of these breed differences (27).

Thus, removing vitamin A from the diet or restricting its intake in the present experiment increased IMF and brought about significant increases in MUFA. Despite these differences, no significant differences were recorded in LW gain or rib fat depth, suggesting a repartitioning of fat throughout the body under different intakes of vitamin A.

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Differential Effect of Lysophospholipids on Activities of Human Plasma Paraoxonase1, Either Soluble or Lipid-Bound

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ABSTRACT: Interaction of paraoxonase1 (PON1) with lysophospholipids was examined with respect to activity regulation and binding property. Paraoxonase activity of purified PON1 was partially inhibited by palmitoyl-lysophosphatidylglycerol (palmitoyl-lysoPG) and lysophosphatidylinositol (lysoPI), which had a stimulatory effect on arylesterase and diazoxonase activities. The selective inhibition of paraoxonase activity by palmitoyl-lysoPG, characterized by noncompetitiveness and charge interaction, was also observed with HDL- or dimyristoylphosphatidylcholine (DMPC)-bound PON1. Meanwhile, lysophosphatidylcholine (lysoPC) stimulated all three activities of purified PON1, although it stimulated DMPC-bound or HDL-bound PON1 to a lesser extent. The stimulatory action of lysophospholipids was observed around their CMC, suggesting that micelle formation of lysophospholpids might be involved in the stimulation of PON1 activity. Presumably in support of this, the tryptophan fluorescence intensity of PON1 was increased by lysophospholipids at concentrations required for the stimulation of PON1 activity. Separately, lysoPC stimulation was less remarkable for DMPC-bound PON1 than for either dimyristoylphosphatidylserine (DMPS)- or dimyristoylphosphatidylglycerol-bound PON1, suggesting a tight association between PON1 and DMPC. In support of this, the stimulatory role of apolipoprotein A-I was less prominent for DMPC-bound PON1 than for DMPS-bound PON1. Taken together, these data suggest that the inhibition of paraoxonase activity by lysoPG or lysoPI may be due to binding to a site distinct from the active center, whereas the stimulation by lysophospholipid may be ascribed to the micelle formation around the lipid-associable region of PON1.

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Paraoxonase1 (PON1), which is bound to HDL, has the ability to hydrolyze organophosphate compounds, carboxylic acid esters, and some lactone compounds (1–7). In addition to interest in a potential use of PON1 in detoxification of toxic organophosphates (5,8), interest in the enzyme has arisen from the idea that PON1 may exert antiatherogenic properties through antioxidant action (3,9–12). In animal and human model experiments, a lower serum level of PON1 was suggested to be associated with an increased risk of atherosclerosis (10,13–16). However, recent findings (17,18) do not support this theory of antioxidant activities of PON1. Nonetheless, PON1 was found to inhibit macrophage cholesterol biosynthesis and atherogenesis, probably through its phospholipase-A2–like activity (11), and also to inhibit oxidized LDL-induced monocyte chemoattractant protein-1 production in endothelial cells (12). Further, PON1 was reported to enhance HDL-mediated macrophage cholesterol efflux via ATP-binding cassette A1 transporter in association with increased HDL binding to the cells (19). Very recently (20), the antiatherogenic properties of HDL-associated PON1 was proposed to involve lysophosphatidylcholine (lysoPC) release in macrophages. Thus, it has been of much interest to clarify whether and/or how the PON1 molecule interacts with lipoproteins or lipid membranes. Although earlier studies demonstrated the stimulation of PON1 arylesterase activity by phospholipids or lysoPC (21–23), the structural relationship for the interaction of PON1 with various lipids has not been established.

Two substrates, paraoxon and phenylacetate, have been employed in the routine assay of PON1 activity (1,4). However, it has been suspected that the binding sites for the two substrates may not overlap exactly with each other (24,25). Furthermore, PON1 exhibits a substrate-dependent polymorphism; the Q(A) isozyme and the R(B) isozyme impart different catalytic activity toward paraoxon, in contrast to the same rate for phenylacetate hydrolysis (5,15,19,26). In support of this, a recent structural analysis indicates that the paraoxon-binding subsite may be not identical to the phenylacetate-binding subsite (27). Somewhat consistent with these findings, there have been reports on greater loss of paraoxonase activity, compared to arylesterase activity, in sera of aging persons or patients with oxidative stress-related diseases (28–33). Our recent data demonstrated that monoenoic acids such as oleic acid and palmitoleic acid caused a preferential inhibition of paraoxonase activity of PON1 (34), in contrast to polyunsaturated or saturated FA, which inhibit both activities to a similar extent (34,35). Also, monooleoylated lysophosphatidylglycerol (lysoPG) demonstrated a selective inhibition of paraoxonase activity, in contrast to a slight stimulation of paraoxonase activity by monooleoylated lysoPC (34). Thus, the structural requirement of lysophospholipids for the inhibition or stimulation of PON1 activities

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Abbreviations: apo A-I, apolipoprotein A-I; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DMPS, dimyristoylphosphatidylserine; DPPC, dipalmitoyl phosphatidylcholine; lysoPC, lysophosphatidylcholine; lysoPG, lysophosphatidylglycerol; lysoPI, lysophosphatidylinositol; lysoPS, lysophosphatidylserine; PON1, paraoxonase1.

needs further study. Furthermore, there is no further information on the effects of lysophospholipids on the activities of PON1 bound to lipid bilayer or HDL, or the cooperative roles between lysoPC and apolipoprotein A-I (apo A-I) in the regulation of lipid-bound PON1 activity.

In this study, we examined the effect of various lysophospholipids on activities of PON1, either soluble or phospholipid-bound, and proposed the possible mechanisms responsible for the inhibitory or stimulatory action of lysophospholipids.

EXPERIMENTAL PROCEDURES

Chemicals. All materials including apo A-I (human plasma) were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Diazoxon was from Chem Service (West Chester, PA). Phospholipids and lysophospholipids were provided by Avanti Polar Lipids, Inc. (Alabaster, AL).

Purification of PON1. PON1 was purified using human plasma from normolipidemic volunteers according to a slight modification of the published procedures (1,22,35,36). Briefly, PON1 was purified from human plasma by (pseudo)affinity chromatography using Cibacron Blue 3GA, DEAE-Sephacel chromatography, Sephacryl S-200-HR gel chromatography, and finally affinity chromatography using concanavalin A–Sepharose. The purified PON1 (740-fold purification), expressing a specific activity of 1251 mmol/min/mg protein and 781 nmol/min/mg protein in the hydrolysis of phenylacetate and paraoxon, respectively, was found to belong to phenotype A type (ratio approximately 1.6) on the basis of the dual substrate method (24).

Assay of PON1. Unless otherwise noted, arylesterase activity of PON1 was determined by monitoring the change of absorbance at 270 nm in 0.5 mL of 50 mM Tris buffer (pH 7.4 + 1 mM $CaCl_2$) containing 1 mM phenylacetate, except the purification procedure, where 10 mM phenylacetate was used (1,22,35). Paraoxonase activity and diazoxonase activity were determined by monitoring the change of absorbance at 405 nm in the same buffer containing 1 mM paraoxon (22), and the change of absorbance at 270 nm in the same buffer containing 1 mM diazoxon (5), respectively. One unit of arylesterase or diazoxonase activity is expressed as 1 μmol of product generated from substrate per min, and 1 unit of paraoxonase activity as 1 nmol of *p*-nitrophenol produced from paraoxon per min.

Preparation of HDL. Serum HDL was isolated from fasted normolipidemic human volunteers by ultracentrifugation at a density ranging between 1.063 and 1.121 g/mL as previously described (9), and dialyzed against 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 1 mM Ca^{2+} at 4°C.

Preparation of liposome and liposome-bound PON1. For the preparation of liposome, each phospholipid, dissolved in chloroform/methanol, was evaporated in a glass vial under vacuum, and suspended in 0.4 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM $CaCl_2$ using a dismembrator (Branson sonifier 250). For the preparation of liposome-associated PON1 (21), A-type PON1 (75 arylesterase units/mL) in the buffer (0.6 mL) containing 1 mM $CaCl_2$ was added to the lipid suspension, and the mixture was incubated under stirring for 4 h at room temperature. Finally, the mixture was centrifuged (100,000*g*, 90 min), and the pellet was washed with the same buffer containing 1 mM $CaCl_2$ to remove the remaining soluble enzyme. The recovery of PON1 activity was estimated to be approximately 50%.

Effect of lysophospholipids on PON1 activities. Purified PON1 was incubated with phenylacetate (1 mM), paraoxon (1 mM), or diazoxon (1 mM) in the presence of each lysophospholipid of various concentrations in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C. One arylesterase unit /mL (0.8 μg protein/mL) of PON1 was used for the hydrolysis of phenylacetate or diazoxon, and 7.5 arylesterase units/mL (6 μg protein/mL) of PON1 was used for paraoxon hydrolysis.

Effect of lysophospholipids on liposome-bound PON1 activities. Liposome-bound PON1 (1.6 arylesterase units/mL) was incubated with phenylacetate (1 mM) or paraoxon (1 mM) in the presence of each lysophospholipid of various concentrations in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C.

Effect of lysophospholipids on HDL-bound PON1 activities. HDL was incubated with 1 mM of phenylacetate or paraoxon in the presence of each lysophospholipid in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C. The activity of PON1 in HDL used was 1 arylesterase unit/mL for arylesterase activity determination and 7.5 arylesterase unit/ml for paraoxonase activity determination. Separately, 100 μl of HDL (8 mg protein/mL) was preincubated with bee PLA_2 (1.5 unit) in 0.4 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C for 30 min, and then the aliquot (1 μL) was taken into 0.5 mL of the above buffer to determine activity of arylesterase and paraoxonase in the presence of palmitoyl-lysoPC.

Effect of apo A-I on arylesterase activity of liposome-bound PON1. Liposome-bound PON1 (1.6 arylesterase units/mL) was preincubated with apo A-I in 0.1 mL of 10 mM PBS buffer (pH 7.4) containing 1 mM Ca^{2+} at 37°C for 30 min, and then the aliquot was taken for the assay of arylesterase activity in the presence or absence of palmitoyl-lysoPC.

Effect of apo A-I on arylesterase activity of PON1 exposed to dimyristoylphosphatidylserine (DMPS). Purified PON1 (2.5 arylesterase units) was preincubated with sonicated DMPS (0.2 mM) in 0.1 mL of the above buffer containing 1 mM Ca^{2+} at 25°C for 5 min. Then apo A-I was added to the mixture. After another10 min incubation, the aliquot was taken for the assay of remaining activity in the presence or absence of palmitoyl-lysoPC.

Kinetic analysis of inhibition of PON1 paraoxonase activity by palmitoyl-lysoPG. PON1 (7.5 arylesterase units) was incubated with paraoxon of various concentrations in the presence of palmitoyl-lysoPG (3 or 10 μM) in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+}, and the inhibition was analyzed by Lineweaver-Burk plot as described previously (35).

Other analyses. Fluorescence emission spectra of PON1 were monitored using a PerkinElmer 550-55 fluorimeter at room temperature (37) with excitation wavelength at 280 nm and emission wavelength at 340 nm (slit width 5 nm). Change in fluorescence was calculated according to the following equation: the relative change of fluorescence = $(I_x - I_o)/I_o$, where I_x is the fluorescence intensity of PON1 in the presence of lysophospholipid and I_o is the fluorescence intensity of PON1 in the absence of lysophospholipid.

RESULTS

Selective inhibition of PON1 paraoxonase activity by lysoPG or lysophosphatidylinositol (lysoPI). Previously (34), oleoylated lipids such as oleic acid or monooleoyl-lysoPG had been reported to partially inhibit PON1 paraoxonase activity. However, the structural requirement of lysophospholipids for the regulation of PON1 activities was not established. First, to see the structural requirement for the inhibition of paraoxonase activity by lysoPG, paraoxonase activity of purified PON1 was determined in the presence of lysoPG with various acyl chains (C_{14}–C_{18}). As demonstrated in Figure 1, the most potent was palmitoyl-lysoPG, which had a maximal inhibition (I_{max}) of 22%, followed by myristoyl-lysoPG (I_{max} = 18%) and stearoyl-lysoPG (I_{max} = 16%). Myristoyl-lysoPG was almost equipotent to oleoyl-lysoPG (I_{max} = 18%). When the dose-dependent effect of palmitoyl-lysoPG, the most potent, on paraoxonase activity was examined (Fig. 2A), the 50% maximal inhibitory concentration of palmitoyl-lysoPG was estimated to be ~3 μM, lower than its CMC value (38,42). In contrast, palmitoyl-lysoPG stimulated arylesterase and diazoxonase activities (Fig. 2A). It is noteworthy that the stimulatory action of palmitoyl-lysoPG tended to increase up to 30 μM, higher than its CMC value (38), and palmitoyl-lysoPG at 30 μM enhanced arylesterase and diazoxonase activities by 25% and 35%, respectively ($P < 0.01$). Thus, palmitoyl-lysoPG was found to be a selective inhibitor of paraoxonase activity of PON1, somewhat different from oleic acid, a preferential inhibitor of paraoxonase activity (34). In a separate study, in which three PON1 activities were examined in the presence of liver lysoPI, it was also found (Fig. 2A) that liver lysoPI (10 μM) inhibited paraoxonase activity partially (24%), while the activities of arylesterase and diazoxonase were augmented slightly (9–14%) by 10 μM lysoPI ($P < 0.05$). Meanwhile, negatively charged lipids such as lysophosphatidylserine (lysoPS) or phosphatidic acid did not inhibit paraoxonase activity.

Binding site for inhibitory palmitoyl-lysoPG. Although the inhibitory action of palmitoyl-lysoPG was exerted at relatively low concentrations with a 50%-maximum inhibitory concentration of approximately 3 μM, the maximal inhibition degree was restricted to ~22% (Fig. 2A). Moreover, the inhibition degree (~22%) did not differ significantly according to the paraoxon concentration (0.25–2.0 mM) in repeated experiments. When the marginal inhibition was subjected to Lineweaver-Burk plot analysis, palmitoyl-lysoPG (3 μM or 10 μM) appeared to express a mixed type of noncompetitive pattern in the inhibition of paraoxonase activity (Fig. 3), supporting the notion that the inhibitory action of palmitoyl-lysoPG might be exerted at a site different from the active center of PON1. From kinetic analysis, the K_i value of palmitoyl-lysoPG is estimated to be 41.2 ± 3.8 μM, much smaller than the K_m value (1.42 ± 0.2 mM) of substrate. Moreover, the inhibitory action of palmitoyl-lysoPG on paraoxonase activity, time-independent, was almost fully (>90%) suppressed by 1 M NaCl, in contrast to no suppressive effect of salt on the stimulation of arylesterase activity by palmitoyl-lysoPG (Park, C.-H., *et al.*, unpublished data).

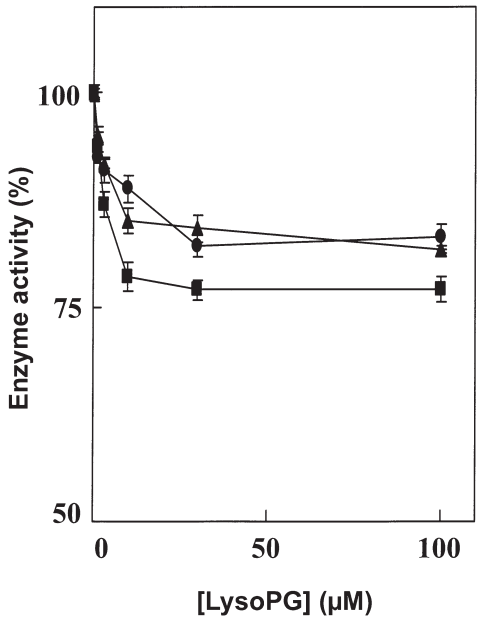

FIG. 1. Inhibitory effect of lysophosphatidylglycerol (lysoPG) on paraoxonase activity of purified paraoxyonasel (PONI). Purified PON1 (7.5 arylseterase unit/mL), A type, was incubated with paraoxon (1 mM) in the presence of lysoPG (1–100 μM) in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C, and the formation of product was monitored at 405 nm as described previously (22). ▲, stearoyl-lysoPG; ■, palmitoyl-lysoPG; ●, myristoyl-lysoPG. Data are expressed as a mean ± SD (bar) value of triplicate assays, presented as a relative value (%) of control activity, which was obtained in the absence of lysophospholipids.

Stimulation of PON1 activities by lysophospholipids. When the effect of various lysophospholipids (10 μM) on arylesterase activity was examined, it was found that palmitoyl-lysoPC, liver lysoPI, and monooleoyl-lysoPS enhanced arylesterase activity by 47%, 10%, and 8%, respectively. In addition, nonionic detergents such as monolaurylglycerol (1–10 mM), Tergitol-10 (1–100 μM), or Triton X-100 (1–100 μM) showed no significant stimulation (<5%) of arylesterase activity. Thus, palmitoyl-lysoPC was the most efficient in stimulating arylesterase activity. The stimulatory effect of lysophospholipids on three PON1 activities, arylesterase, paraoxonase, and diazoxonase, was investigated in more detail. In further study of the effect of palmitoyl-lysoPC on these three activities (Fig. 2B), it was found to express a concentration-dependent increase of all three activities up to 30

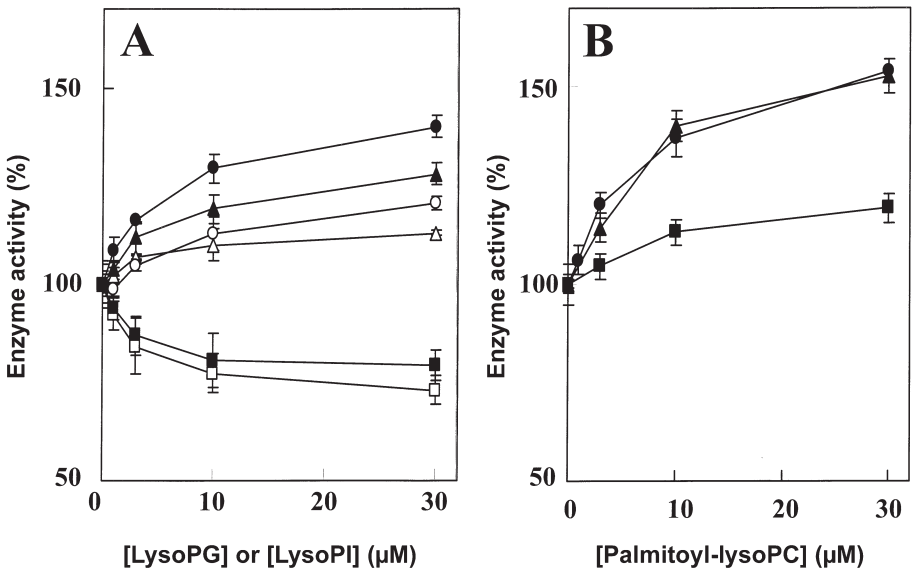

FIG. 2. Inhibitory effect of lysophospholipids on activities of purified PON1. (A) Purified PON1 was incubated with 1 mM of phenylacetate (triangle), paraoxon (rectangle), or diazoxon (circle) in the presence of palmitoyl-lysoPG (filled symbols) or liver lysophosphatidylinositol (lysoPI) (open symbols) in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C. (B) The same experiment was done in the presence of palmitoyl-lysophosphatidylcholine (lysoPC). One arylesterase unit/mL was used for assay of the hydrolysis of phenylacetate and diazoxon, whereas 7.5 aryleterase units/mL were used for assay of hydrolysis of paraoxon. Data are expressed as a mean ± SD (bar) value of triplicate assays, presented as a relative value (%) of control activity, which was obtained in the absence of lysophospholipids. Control activity was ΔO.D. 0.081/min for paraoxonase activity, ΔO.D. 0.37/min for arylesterase activity, and ΔO.D. 0.35/min for diazoxonase activity. For abbreviations see Figure 1.

μM, above its CMC value. At concentration as low as 10 μM, palmitoyl-lysoPC stimulated the activities of arylesterase, paraoxonase, and diazoxonase by 47 %, 14%, and 40%, respectively. Thus, arylesterase and diazoxonase activities were more sensitive to the palmitoyl-lysoPC stimulation, compared to paraoxonase activity ($P < 0.05$).

Effect of lysophospholipids on Trp fluorescence of PON1. According to a previous report (27), PON1 is known to contain three helical regions, of which two helices (H1 and H2) are associable with amphiphilic phospholipids. Since the maximal stimulatory effect of lysophospholipids on arylesterase activity was observed at or above their CMC values (Fig. 2), the lysophospholipid stimulation was thought to be due to the micelle formation around some polypeptide regions, probably helical regions. Actually, three of four Trp residues reside in or close to helical regions (H2 and H3) of PON1. Therefore, the effect of lysophospholipids on the fluorescence spectrum of Trp residues in PON1 was examined. As demonstrated in Figure 4, each lysophospholipid enhanced Trp fluorescence intensity of PON1 in a concentration-dependent manner up to 30 μM with a sharp inflection at 10 μM, suggesting that some Trp residue–containing regions of PON1 might be exposed to a relatively low level of lysophospholipids. In contrast, Triton X-100, an amphiphile, failed to enhance PON1 Trp fluorescence even above its CMC value (Fig. 4A). A related study indicates that lipids, which show an enhancing effect on PON1 Trp fluorescence, seem to stimulate arylesterase activity effectively (Nguyen, S.D., *et al.*, unpublished data). The concentration-dependent effect of lysophospholipids on the fluorescence spectrum indicates that the concentration of lysophospholipids required for a remarkable increase in Trp fluorescence intensity is close to that for

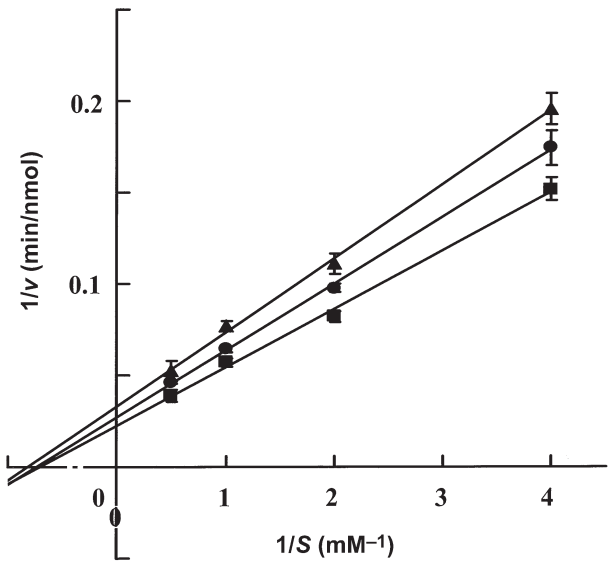

FIG. 3. Lineweaver-Burk double-reciprocal plot for partial inhibition of paraoxonase activity by palmitoyl-lysoPG. PON1 (7.5 arylesterase units/mL) was incubated with paraoxon of various concentrations (0.25–2 mM) in the presence or absence of palmitoyl-lysoPG (3 or 10 μM) in 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C. ■, control; ●, 3 μM ; ▲, 10 μM. For abbreviations see Figure 1.

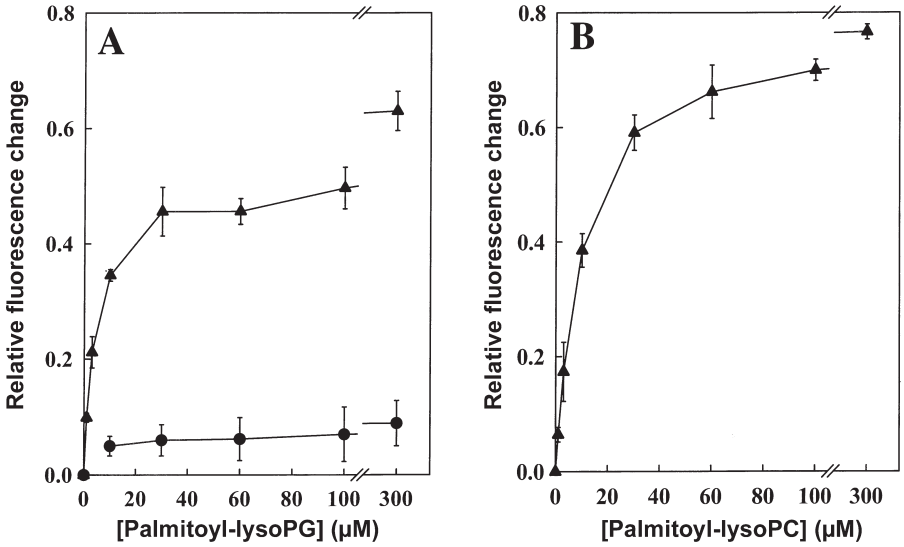

FIG. 4. Effect of lysophospholipids on the fluorescence of PON1. (A) PON1 (6 arylesterase units; 4.8 mg) was incubated with palmitoyl-lysoPG at various concentrations (0, 1, 3, 10, 30, 60, 100, or 300 μM) in 1 mL Tris buffer (pH 7.4) containing 1mM Ca^{2+}. Triton X-100 was used as control (filled circle). (B) The same experiment was done in the presence of palmitoyl-lysoPC. Fluorescence emission spectra of PON1 were monitored using a PerkinElmer 550-55 fluorimeter at room temperature, with the excitation wavelength at 280 nm and the emission wavelength at 340 nm (slit width 5 nm). Change in fluorescence was calculated as described in this paper. Data are expressed as a mean ± SD (bar) value of triplicate assays. For abbreviations see Figures 1 and 2.

the stimulation of PON1 arylesterase activity (Fig. 2). These data indicate that the stimulatory action of lysophospholipids is due to their association around the polypeptide region containing at least one Trp residue.

Effect of lysophospholipids on liposome-bound PON1. Previous studies showed that anchoring of the PON1 N-terminus to phospholipids was important for PON1 activity (21,23). Therefore, the prior binding of the N-terminus to phospholipid micelles was expected to result in the decreased response of PON1 to further stimulation by lysophospholipid. In this regard, the effect of lysophospholipids on dimyristoylphosphatidylcholine (DMPC) liposome– or DMPS liposome–bound PON1 activities was investigated. As shown in Fig. 5A, DMPC liposome–bound paraoxonase activity was inhibited partially (approximately 22%) by palmitoyl-lysoPG (10 μM), and the inclusion of 1 M NaCl restored the lost activity to 93% of control level, similar to the finding with purified PON1. Arylesterase activity of DMPC-bound PON1 was not altered remarkably by palmitoyl-lysoPG (1–10 μM), contrasting with the finding for purified PON1 arylesterase (Fig. 2A). Thus, DMPC-anchored arylesterase was resistant to palmitoyl-lysoPG stimulation. Furthermore (Fig. 5A), palmitoyl-lysoPG inhibited DMPS-bound paraoxonase activity by 43% while stimulating arylesterase activity by 28% ($P < 0.05$). Thus, the effect, inhibitory or stimulatory, of palmitoyl-lysoPG on PON1 activities seems to be more remarkable for DMPS-bound PON1, compared to DMPC-bound PON1.

When the effect of palmitoyl-lysoPC on liposome-bound PON1 activities was examined (Fig. 5B), palmitoyl-lysoPC (10 μM) enhanced DMPC-bound paraoxonase activity and DMPC-bound arylesterase activity by 13% and 23%, respectively ($P < 0.05$). Thus, compared to purified PON1 activities, DMPC-anchored PON1 activities were less sensitive to the stimulatory effect of palmitoyl-lysoPC, reflecting that the polypeptide regions of PON1 that are associable with palmitoyl-lysoPC were already occupied by DMPC. In support of this, PON1 associated with dipalmitoyl phosphatidylcholine (DPPC), which is more nonpolar, was less responsive to palmitoyl-lysoPC than DMPC-associated PON1. Further, palmitoyl-lysoPC (10 μM) augmented DMPS-bound paraoxonase and arylesterase activities by 41% and 83%, respectively; these increases are much greater than the values achieved with purified PON1.

Effect of lysophospholipids on HDL-bound PON1. The effect of lysophospholipids on HDL-bound PON1 activities was examined. As demonstrated in Fig. 6A, palmitoyl-lysoPG at relatively low concentrations (1–10 μM) inhibited HDL-paraoxonase activity with a maximal inhibition of ~15% ($P < 0.05$), but it had no remarkable effect on HDL-associated arylesterase activity. A similar result was also observed with soybean lysoPI. Thus, palmitoyl-lysoPG or liver lysoPI inhibited HDL-associated PON1 paraoxonase activity selectively, similar to the finding with purified PON1. Separately (Fig. 6B), palmitoyl-lysoPC (30 μM) was observed to enhance HDL-bound arylesterase activity by ~13% ($P < 0.05$), but showed no effect on HDL-paraoxonase activity, indicating that HDL-associated PON1 was less responsive to palmitoyl-lysoPC compared to DMPC-bound PON1. Accordingly, it was supposed that the polypeptide regions of PON1 that are associable with palmitoyl-lysoPC might be intimately

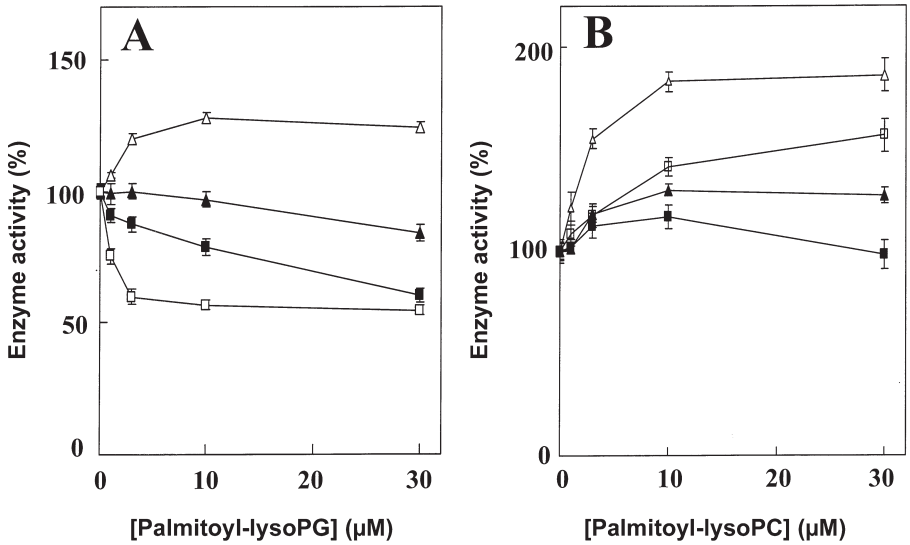

FIG. 5. Effect of lysophospholipids on activities of liposome-bound PON1. (A) Liposome-bound PON1 was incubated with 1 mM of phenylacetate or paraoxon in the presence of palmitoyl-lysoPG (1–30 μM) in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C. (B) The same experiment was done in the presence of palmitoyl-lysoPC. The amount of dimyristoylphosphatidylcholine (DMPC) (filled symbols) or dimyristoylphosphatidylserine (DMPS) (open symbols)-bound PON1 used is 0.5 arylesterase unit/mL for the hydrolysis of phenylacetate (triangle) and 3 arylesterase units/mL for paraoxon hydrolysis (rectangle). Data are expressed as a mean ± SD (bar) value of triplicate assays, presented as a relative value (%) of control activity, which was obtained in the absence of lysophospholipids. For abbreviations see Figures 1 and 2.

bound to HDL lipid. In an attempt to test this possibility, HDL was pretreated with bee PLA_2 to generate phosphatidylcholine-deficient HDL. Then, the PLA_2-pretreated HDL, after 500-fold dilution, was subjected to the stimulation by palmitoyl-lysoPC. The prior treatment of HDL with PLA_2 led to ~47% loss of arylesterase activity, and the subsequent exposure of PLA_2-pretreated HDL to palmitoyl-lysoPC (30 μM) recovered > 95% of the lost activity (Fig. 6B). Likewise, the prior treatment of HDL with PLA_2 led to ~50% loss of paraoxonase activity, and the subsequent treatment with palmitoyl-lysoPC (30 μM) recovered > 90% of the lost activity. Thus, the site responsible for palmitoyl-lysoPC stimulation seems to be occupied by phosphatidylcholine in HDL.

Effect of apo A-I on arylesterase activity of PON1 associated with phospholipids. Earlier (21,23), PON1 N-terminus had been reported to bind to phosphatidylcholine optimally presented in association with apo A-I. Therefore, it was supposed that activity of HDL-bound PON1 might be maximally expressed by phosphatidylcholine recruited by apo A-I. To test this, the effect of apo A-I, in combination with lysoPC, on arylesterase activity of PON1 bound to DMPS, dimyristoylphosphatidylglycerol (DMPG), or DMPC was examined. When DMPS-bound PON1 was preincubated with apo A-I for 30 min before the determination of arylesterase activity (Fig. 7A), the arylesterase activity was enhanced by apo A-I (0.3–10 μM) with a maximal stimulation of 174%. The preincubation with apo A-I followed by palmitoyl-lysoPC (10 μM) further augmented PON1 arylesterase activity up to 210%, higher than the levels achieved with apo A-I alone (174%) or 10 μM palmitoyl-lysoPC alone (178%), which suggests that apoA-I and palmitoyl-lysoPC might cooperate to maximize the arylesterase activity when they are bound to DMPS. A similar result was also observed with DMPG-bound arylesterase activity, although to a lesser extent. Meanwhile, the stimulatory effect of apo A-I (10 μM) on DMPC-bound arylesterase activity was restricted to 19%, which is less than the effect (25%) achieved with palmitoyl-lysoPC alone (10 μM). In comparison, DMPS-bound arylesterase (74% stimulation) was much more responsive to apo A-I stimulation or palmitoyl-lysoPC stimulation than DMPC-bound arylesterase (19% stimulation), leading to the assumption that arylesterase activity of PON1 may not be fully expressed in DMPS liposome. In related experiment to address this notion (Fig. 7B), the preincubation of PON1 with DMPS (200 μM) resulted in 47% loss of arylesterase activity, and the subsequent inclusion of apo A-I (1–10 μM) in the mixture containing PON1 and DMPS (200 μM) restored the lost activity with a full recovery (100% of control). Further, the inclusion of palmitoyl-lysoPC (10 μM), in combination with apo A-I (≥ 3 μM), augmented the activity up to 140%, far beyond that (100%) achieved with apo A-I alone. Similarly, the preincubation of PON1 with DMPS (200 μM) led to 20% loss of paraoxonase activity, and the subsequent inclusion of apo A-I (1–10 μM) in the mixture restored the lost activity with a full recovery. Further, the inclusion of palmitoyl-lysoPC (10 μM), in combination with apo A-I (3–10 μM), augmented the activity to

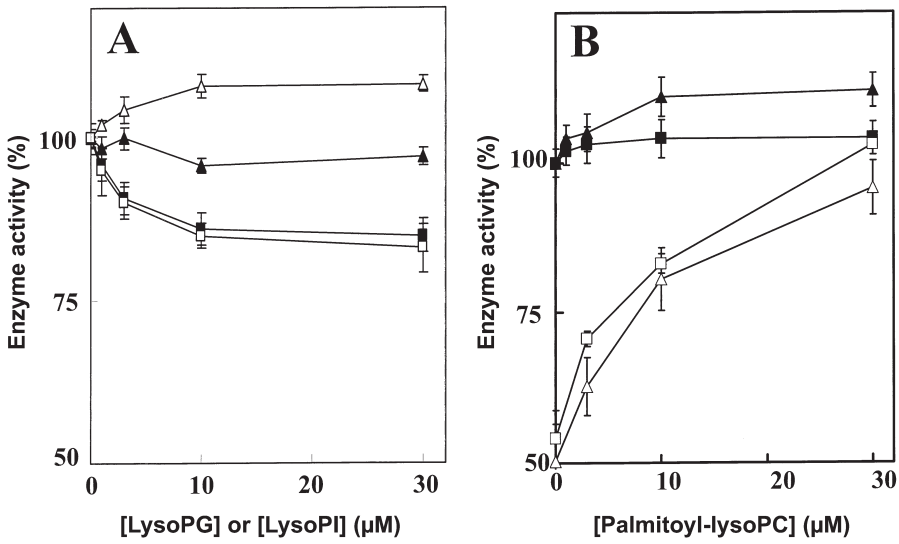

FIG. 6. Effect of lysophospholipids on HDL-associated PON1 activities. (A) HDL was incubated with 1 mM of phenylacetate, diazoxon, or paraoxon in the presence of palmitoyl-lysoPG (filled symbols) or lysoPI (open symbols) in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C. The activity of PON1 in HDL used is 1 arylesterase unit/mL for arylesterase activity (triangle) and 7.5 arylesterase unit/mL for paraoxonase activity (rectangle). (B) HDL was incubated with 1 mM of phenylacetate or paraoxon in the presence of palmitoyl-lysoPC in the same buffer. Data are presented as a relative value (%) of control activity, which was obtained in the absence of lysophospholipids. The activity of PON1 in HDL used is 1 arylesterase unit/mL for arylesterase activity (filled triangle) and 7.5 arylesterase unit/mL for paraoxonase activity (filled rectangle). Separately, 100 μl of HDL (8 mg protein/mL) was preincubated with bee PLA_2 (1.5 unit) in 0.5 mL of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca^{2+} at 25°C for 30 min, and then an aliquot (1 μL) was taken into 0.5 mL of the same buffer containing palmitoyl-lysoPC (0–30 μM) for the determination of arylesterase activity (open triangle) and paraoxonase activity (open rectangle). Data are expressed as a mean ± SD (bar) value of triplicate assays, presented as a relative value (%) of control activity of HDL, which was preincubated in the absence of PLA_2. For abbreviations see Figures 1 and 2.

120%. In a separate experiment, the preincubation of PON1 with DMPC (200 μM) led to a 30% increase of arylesterase activity, and the subsequent inclusion of apo A-I (1–10 μM) in the mixture failed to further enhance the activity. Also, no further increase was observed with the combination of palmitoyl-lysoPC (10 μM) and apo A-I (3–10 μM).

DISCUSSION

There have been reports on the interaction between PON1 molecule and lipids (9,21,23,34,35,39). Our present data may add to the knowledge concerning the interaction of PON1 with phospholipids as well as lysophospholipids. From the arguments over the phospholipase-like activity of PON1 solutions (17–20), some researchers question whether the potential phospholipase activity of PON1 might influence the present results. However, this possibility is excluded as a result of the findings that purified PON1 used here did not hydrolyze DMPC, and that the stimulation of PON1 arylesterase activity by DMPC was not influenced by PAF hydrolase inhibitor (Nguyen, S.D., *et al.*, unpublished data).

The effect, inhibitory or stimulatory, of lysophospholipids on PON1 activity depends on their structure. Moreover, the effect of negatively charged lysophospholipids on PON1 activities differed according to the type of substrate; lysoPG or lysoPI inhibited paraoxonase activity partially, but stimulated arylesterase and diazoxonase activities, representing a selective inhibition of paraoxonase activity. This may support the notion that the paraoxon-binding subsite does not coincide with the phenylacetate-binding subsite (27,40). Further, the possibility that the selective inhibition of paraoxonase activity by lysoPG or lysoPI may occur at a site different from the active center may be suggested by the noncompetitive nature of the partial inhibition of paraoxonase activity by palmitoyl-lysoPG.

One structural feature of lysophospholipids related to the inhibition of paraoxonase activity is their negatively charged regions with bases such as glycerol or inositol. In addition, the size of the acyl chain in lysoPG is important; the palmitoyl group is the most effective. Meanwhile, an oleoyl group is not necessary for the selective inhibition of paraoxonase activity, which is somewhat different from previous suggestion (34). The inhibitory action of lysoPG at concentrations below its CMC value, a characteristic feature expressed by a monomer portion of amphiphiles (38,41,42), implicates palmitoyl-lysoPG monomer in such an inhibition. In addition, there is no relationship between the inhibitory potency of lysoPG series and their CMC values (41,42). Moreover, the inhibitory action of palmitoyl-lysoPG was suppressed by 1 M NaCl, contrary to the promoting effect of high salt concentration on micelle formation (38,41). Such a selective inhibition

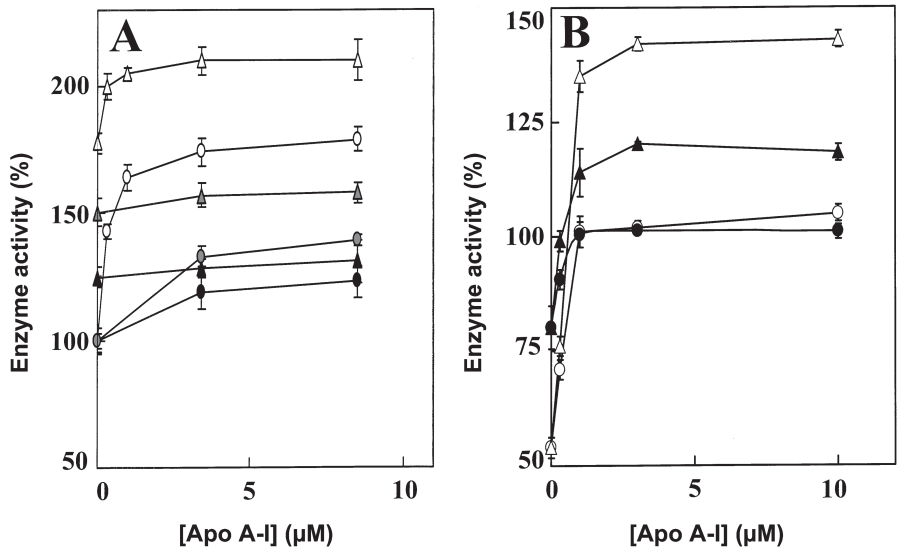

FIG. 7. Effect of apolipoprotein A-I (apo A-I) on PON1 activities associated with phospholipids. (A) Liposome-bound PON1 (1.6 arylesterase unit/mL) was preincubated with apo A-1 (0.3–8.5 μM) in 0.1 mL of 10 mM PBS buffer (pH 7.4) containing 1 mM Ca^{2+} at 37°C for 30 min, and an aliquot was taken for the assay of arylesterase activity in the presence (triangle) or absence (circle) of palmitoyl-lysoPC (10 μM). DMPC (filled symbols); DMPS (open symbols); dimyristoylphosphatidylglycerol (DMPG) (shaded symbols). (B) Purified PON1 (2.5 arylesterase units/mL) was preincubated with DMPS (0.2 mM) in 0.1 mL of the same buffer containing 1 mM Ca^{2+} at 25°C for 5 min, and then apo A-1 (0.3–10 μM) was added to the mixture. After further incubation for 10 min, aliquots were taken for the assay of arylesterase activity (open symbol) and paraoxonase activity (filled symbol) in the presence (triangle) or absence (circle) of palmitoyl-lysoPC (10 mM). Data are expressed as a mean ± SD (bar) value of triplicate assays, presented as a relative value (%) of control activity, which was preincubated in the absence of phospholipid. For abbreviations see Figures 1, 2, and 5.

by palmitoyl-lysoPG was also observed with HDL- or DMPC-bound paraoxonase activity in studies where the N-terminal sequence of PON1 was anchored to phospholipids (21). In this respect, the N-terminal sequence of PON1 molecule is not likely implicated in the inhibition of paraoxonase activity by palmitoyl-lysoPG.

Previous reports (21,23,27) have demonstrated that the anchoring of the PON1 N-terminus to lipid is important for the maintenance of PON1 arylesterase activity. The same mechanism may account for the stimulation of PON1 arylesterase activity by some phosphatidylcholines (23,27). In agreement with this, the stimulatory action of palmitoyl-lysoPC was the more remarkable for purified PON1 than for DMPC-bound PON1 and HDL-associated PON1. In further support, the stimulatory effect of palmitoyl-lysoPC was still less pronounced in PON1 associated with DPPC, which is more nonpolar than DMPC. This might explain why PON1 activity in PLA_2-modified HDL, which is phosphatidylcholine-deficient, became more responsive to the palmitoyl-lysoPC stimulation than HDL-associated PON1. However, a recent study (27) indicates that besides the N-terminal signal sequence, the H1 helix, H2 helix, and its loop, which lie in the interphase between the HDL hydrophobic interior and exterior aqueous phase, also contribute to the association of PON1 with HDL lipids. Further, a very recent report (43) indicates that there is no noticeable difference of specific arylesterase activity between truncated variant PON1 devoid of the N-terminal 20 amino acid residues bound to apo A-I rHDL and intact PON1 bound to apo A-I rHDL, which suggests that the N-terminus of PON1 may not be involved in the stimulation mechanism. From this, it is conceivable that the stimulation of PON1 by lipids may be due to their binding to the interphase of PON1, which is associable with lipids (27). The possible interaction between lysophospholipid and the PON1 interphase may be implied from the enhancing effect of lysophospholipids on the fluorescence intensity of Trp residues in the PON1 molecule. PON1 contains four Trp residues, with three of these being placed in or close to the interphase region (22,27). Probably in support of this, the stimulation of PON1 activity by lysophospholipids was observed at their concentrations corresponding to those required for the enhancement of the Trp fluorescence.

Earlier studies showed that the efficient binding of PON1 N-terminal region to HDL required the coexistence of apo A-I (21,23,44). Further, the binding of the N-terminal sequence to phospholipids in the presence of apo A-I was crucial for the maximal activity of PON1 (21). Thus, apo A-I might contribute to full expression of PON1 activity by promoting micelle formation of phospholipids around the N-terminal signal sequence and/or the interphase of PON1. This might be extended to explain why PON1 associated with HDL that is rich in apo A-I differed from DMPC liposome–bound PON1 in its response to palmitoyl-lysoPC. The interface of DMPC-bound PON1 is still available for the interaction with phospholipids recruited by apo A-I, whereas the interface of PON1 is already associated with lipids in the HDL particle. The positive effect of apo A-I

or palmitoyl-lysoPC was more remarkable for DMPS-bound PON1 than for DMPC-bound PON1. Further, the stimulating effect of apo A-I or palmitoyl-lysoPC on DMPS-bound arylesterase activity was far beyond their effect on purified PON1 activity. A plausible explanation for this is that apo A-I or palmitoyl-lysoPC may exert an additional effect by selectively binding to the contact region between PON1 and DMPS lipid, and restoring the activity of DMPS-bound PON1, which is in a state of suppressed catalysis.

The low response of DMPC-bound PON1 to apo A-I may be ascribed to the limited role of apo A-I for DMPC-bound PON1 in activated state. However, the stimulation of purified PON1 by palmitoyl-lysoPC was not further enhanced by apo A-I, because palmitoyl-lysoPC alone can maximally stimulate the activity through micelle formation around the N-terminal region and/or the interphase. From the finding that the maximal stimulation of purified PON1 arylesterase activity by palmitoyl-lysoPC is similar to that achieved with the combination of DMPC and apo A-I, it is likely that the same polypeptide region may be implicated in the stimulation of PON1 activity by phospholipids or lysophospholipids.

Taken together, the inhibition of PON1 paraoxonase activity was exhibited by lysoPG and lysoPI, which could bind to a specific site different from the catalytic site, whereas the stimulation of three PON1 activities was expressed commonly by lysophospholipids that are associable with the N-terminal region and/or the interphase. Therefore, paraoxonase activity and arylesterase activity of HDL would be significantly altered in pathological states that are accompanied by PLA_2 hydrolysis of phospholipids (45–49), in which a considerable level of lysophospholipids could be generated. This might be more prominent in atherosclerotic plaques containing proinflammatory stimuli such as lysophosphatidylcholine (50) or in phospholipid-deficient HDL (51). In addition, the distribution or amount of apo A-I in HDL particles (23) might be another factor that affects PON1 activities in HDL. It remains to be clarified in further studies whether or how the possible interaction between PON1 and lysophospholipids can affect the potential antiatherogenic function of PON1 *in vivo*.

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METHOD

Analysis of Triacylglycerols of Seeds and Berries of Sea Buckthorn (*Hippophaë rhamnoides*) of Different Origins by Mass Spectrometry and Tandem Mass Spectrometry

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ABSTRACT: TAG of seeds, berries, and fruit pulp/peel of different subspecies of sea buckthorn (*Hippophaë rhamnoides*) were analyzed by MS and tandem mass spectrometry (MS/MS). The seeds contained mainly TAG with acyl carbon number (ACN) of 52 with 2–6 double bonds (DB) (20–30%), and TAG of ACN 54 with 3–9 DB (70–80%). In the pulp/peel fraction, the major TAG were species with ACN:DB of 48:1 to 48:3 (19–49%), 50:1 to 50:4 (31–41%), and 52:1 to 52:6 (9–19%). The molecular weight species of whole berries largely resembled those of fruit pulp/peel with additional species of ACN 54 from the seeds (5–24%). Subspecies (ssp.) *sinensis* differed from ssp. *mongolica* and *rhamnoides* by having a higher proportion of TAG of ACN 52 (27% vs. 21% and 22%, $P < 0.05$) and a lower proportion of ACN 54 (71% vs. 79% and 78%, $P < 0.01$) in seed TAG. Seed TAG of ssp. *mongolica* contained a higher proportion of more unsaturated species compared with those of the two other subspecies. Berry TAG of ssp. *mongolica* had the highest proportion of molecular species of ACN 48 due to the higher proportion of palmitic and palmitoleic acids and the lower seed content of the berries. Overall, palmitic acid favored the *sn*-1 and *sn*-3 positions. The order of preference of unsaturated FA for the *sn*-2 position depended at least partially on the FA combination of TAG. Seed TAG of ssp. *mongolica* contained a higher proportion of α-linolenic acid in the *sn*-2 position than those of ssp. *sinensis*. In berry TAG, ssp. *mongolica* had the highest proportions of palmitoleic and linoleic acids in the *sn*-2 position, and the lowest proportion of oleic/*cis*-vaccenic acid in the *sn*-2 position, among the three subspecies.

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Sea buckthorn (*Hippophaë rhamnoides* L.) is increasingly recognized as an important potential source of bioactive ingredients for functional foods, nutraceuticals, and personal-care products. Sea buckthorn berries are characterized by a high content of water-soluble antioxidants and vitamins in the berry juice and flesh. The uniqueness of the composition of the sea buckthorn berry also lies in the fact that both the seed and the fruit flesh/peel contain oil. The seeds of sea buckthorn typically contain 6–11% oil, whereas the oil content in the fruit flesh and peel varies considerably from 0.5 to 5% with its origin (1,2). Oils from seeds and fruit flesh/peel of sea buckthorn have clearly distinct FA compositions; seed oil is rich in linoleic (18:2n-6) and α-linolenic (18:3n-3) acids, whereas oil from fruit flesh and peel contains a high level of palmitoleic acid (16:1n-7) (2). In addition, oils from the seeds and the fruit soft parts (flesh/peel) can be differentiated by the high content of carotenoids usually found in the latter (1).

The health effects of sea buckthorn seed oil and pulp oil have been extensively documented in the literature in various languages. Topical use of sea buckthorn oils promotes the healing of wounds, burns, scalds, and different types of dermatitis of the skin (1). Dietary intake of sea buckthorn oils has been shown to increase antioxidation capacity, enhance the function of the immune system, and improve the health of the skin, the mucous membranes, and the cardiovascular system (1,3–5).

As a major class of active compounds, FA play an important role in the nutritional effects of sea buckthorn oils. Both the composition of FA and their distribution in TAG influence the nutritional value of oils and fats. After ingestion of oils, the FA in the secondary (*sn*-2) position of TAG have a different metabolic fate compared with those in the primary (*sn*-1 and *sn*-3) positions, resulting in different nutritional and health effects (6,7).

Sea buckthorn oils have been used in China and Russia as ingredients for health-care products for more than half a century. Health-care and personal-care products based on sea buckthorn oils have become increasingly popular in Western countries. This has resulted in an increased demand for sea buckthorn oil in these countries. Selection of the sea buckthorn oils most suitable for specific applications and maintenance of the stable quality of the oils are extremely challenging due to the great variation in their composition depending on the origin of the berries and the oil production technology. Because of the compositional variation among the oils from different sources, the FA composition alone is not always enough to establish the authenticity of sea buckthorn oil. Structural features of TAG of seed and fruit flesh/peel of sea buckthorn provide valuable information for distinguishing sea buckthorn oils from adulterations.

In the present study, the pattern of molecular weight distribution and regioisomers of TAG of seeds, berries, and fruit

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Abbreviations: ACN, acyl carbon number; DB, double bond; CID, collision induced dissociation; MS/MS, tandem mass spectrometry; MTBE, methyl *t*-butyl ether; ssp., subspecies.

flesh/peel fraction of three major subspecies of sea buckthorn were determined. The aim was to reveal the structural features of TAG common to the species, as well as the differences among the subspecies.

MATERIALS AND METHODS

Berries. Wild berries of *Hippophaë rhamnoides* ssp. *sinensis* were collected from their natural growth sites in Shanxi, Shaanxi, Qinghai, and Gansu provinces of China in 1997. Berries of three cultivars (Ruet, Luchezarnaya, and Dar Katuni) of *H. rhamnoides* ssp. *mongolica* were collected from the research station of the Institute of Botany, Siberian Branch of the Russian Academy of Science, Novosibirsk, Russia, in 1997. Wild berries of *H. rahmnoides* ssp. *rhamnoides* were picked in autumn 1999 from five natural growth sites on the Baltic coast of southwest Finland. The berries were loosely frozen immediately after picking.

Extraction of lipids. Frozen berries were lyophilized to a constant weight before manual separation of seeds from fruit pulp and peel. Oil was extracted from seeds, berries, and the fruit flesh/peel fraction as previously described (2). Briefly, a sample of 1 g was crushed in liquid nitrogen and extracted with 3 × 30 ml of a solvent mixture of chloroform/methanol (2:1, vol/vol). The extract was purified by repeated washing with a 0.88% potassium chloride water solution and methanol/water (1:1, vol/vol) before the solvents were removed using a rotary film evaporator. The total lipids extracted were dissolved in chloroform and stored at –20°C until analyzed.

Separation of TAG from total lipids. Triacylglycerols were isolated from total lipids on a Sep-Pak silica cartridge as previously described (2,8). After evaporation of chloroform to dryness under nitrogen, total lipids were reconstituted in hexane. Total lipids were applied on a preconditioned Sep-Pak silica cartridge. After elution of hydrocarbons, wax esters, and steryl esters with 15 ml hexane/MTBE (methyl *t*-butyl ether) (200:2, vol/vol), TAG were eluted with 20 ml of hexane/MTBE (96:4, vol/vol). After evaporation of solvents under nitrogen, the TAG were dissolved in hexane and stored at –20°C until analyzed.

Analysis of FA composition of TAG. TAG was transesterified by sodium methoxide catalysis (9). The FAME were analyzed by GC using a PerkinElmer (San Jose, CA) Autosystem equipped with a programmed split/splitless injector and a flame ionization detector A silica capillary column NB-351 (length 25 m, i.d. 0.32 mm, d_f 0.2 μm, HNU-Nordion Ltd, Helsinki, Finland) was used for GC analysis. The flow rate, split ratio, and temperature programs for the oven, injector, and detector for GC analysis were as reported previously (2).

Mass spectrometric analysis of molecular weight distribution of TAG. Mass spectrometric analysis of molecular weight distribution was carried out on the TAG of the following samples, four analyses each: seeds of ssp. *sinensis* (from three natural growth sites in Shanxi, Qinghai, and Gansu, China), ssp. *rhamnoides* (from three natural growth sites in Vaasa and Pyhämaa, Finland), and ssp. *mongolica* (three commercial varieties); whole berries of ssp. *sinensis* (from four natural growth sites in Shanxi, Shaanxi, Qinghai, and Gansu, China), ssp. *rhamnoides* (from five natural growth sites in Hirsilahti, Siikajoki, Vaasa, and Pyhämaa, Finland), and ssp. *mongolica* (three commercial varieties); and seedless fruit pulp/peel of ssp. *sinensis* (from one natural growth site in Shanxi, China) and ssp. *rhamnoides* (from one natural growth site in Pyhämaa, Finland).

Molecular weight distribution of TAG was analyzed using a Finnigan (San Jose, CA) MAT TSQ-700 triple-quadrupole mass spectrometer equipped with a Finnigan MAT ICIS II data system. CI with ammonia in negative-ion mode was used according to parameters previously optimized and controlled regularly in the laboratory (10). The pressure of the reactant gas ammonia was 8500 mtorr and the ion source temperature was 200°C in order to produce abundant $[M - H]^-$ ions without formation of interfering fragments. The electron energy was 70 eV and the filament current was 400 μA. Samples were introduced to the ion source on a rhenium wire of a direct exposure probe. A rapid desorption of the sample using a high heating rate (40 mA s^{-1}) was used to minimize the thermal degradation of unsaturated TAG, and hence to eliminate the need for quantitative correction of different molecular weight species (10).

Under the selected analytical condition, $[M - H]^-$ ions were generated from deprotonation of TAG from chemical ionization. The $[M - H]^-$ ions were analyzed by scanning the mass range of *m/z* 500–1000. The calculation program includes automatic correction of the $[M - H]^-$ ions for ^{13}C isotope species; $[M - H]^-$ ion species that are less abundant than the ions that are smaller by two mass units (with one more double bond), in particular, would display significant quantitative error without corrections (10). The relative abundances of TAG with different molecular weights were determined according to the intensity of $[M - H]^-$ ions of different *m/z* values. The numbers of total acyl carbons and of the double bonds in the acyl chains of TAG were calculated according to the *m/z* value of $[M - H]^-$ ions and the FA composition analyzed by GC.

Tandem mass spectrometry (MS/MS) analysis of regioisomers of TAG. Regioisomers of major acyl carbon number: double bond (ACN:DB) species of TAG of the following samples were analyzed by MS/MS, four analyses for each ACN:DB species: seeds of ssp. *sinensis* (from three natural growth sites in Shanxi, Qinghai, and Gansu, China), ssp. *rhamnoides* (from three natural growth sites in Vaasa and Pyhämaa, Finland), and ssp. *mongolica* (three commercial varieties); and whole berries of ssp. *sinensis* (from three natural growth sites in Shanxi, Qinghai, and Gansu), ssp. *rhamnoides* (from three natural growth sites in Hirsilahti and Pyhämaa, Finland), and ssp. *mongolica* (three commercial varieties).

Regioisomers of TAG were analyzed by determining the molecular weights of FA in the primary (*sn*-1 and *sn*-3) positions and the secondary (*sn*-2) position. Negative-ion MS/MS with ammonia CI and argon collision-induced dissociation

(CID) was carried out with a Finnigan MAT TSQ-700 triple-quadrupole mass spectrometer equipped with a Finnigan MAT ICIS II data system (10,11). The conditions for CI were as described in the previous section. The pressure of the collision gas was 1.8 mtorr and the collision energy was 15 eV. TAG samples (1 µl, approximately 1 mg/mL) dissolved in hexane were placed on a rhenium wire at the end of a direct insertion probe. The solvent was allowed to evaporate and the rhenium wire and the probe were introduced directly into the ion source. The probe was heated to 300°C at the maximum heating rate (300°C/min). The scan time for each individual scan was 8 s, and a total of 10–12 spectra were obtained. The first and the last spectra were discarded, and the rest were summed and displayed.

The CI of TAG produced a complex mixture of ions within the ion source. The first quadrupole of the mass spectrometer transmitted ions of one particular *m/z* value. The selected $[M - H]^-$ ions (parent ions) entered the second (RF-only) quadrupole, collided with argon, and were fragmented. The fragment ions were then analyzed by the third quadrupole and displayed in daughter-ion spectra. These CID spectra were used to deduce the structural information about TAG regarding the FA and their distribution in TAG molecules.

The ions $[M - H]^-$, $[RCOO]^-$, and $[M - H - RCOOH - 100]^-$ in the CID spectra were taken into account in the calculation. The *m/z* value of $[M - H]^-$ and the FA composition of TAG determined by GC gave information about the ACN:DB of TAG. The molecular weights of the FA were interpreted according to the *m/z* values of the $[RCOO]^-$ ions. These, together with the results of GC analysis, were used to identify the FA within the specific ACN:DB species of TAG. Empirical correction factors for the abundance of $[RCOO]^-$ ions were used on the basis of regular analysis of reference TAG containing the following FA: 18:0 (1.0); 18:1n-9 (1.1); 18:2n-6 (1.2); 18:3n-3 (1.3); 16:0 (1.1); and 16:1n-7 (1.3). The FA 18:1n-7 was assumed to have the same correction factor as 18:1n-9. Under the selected conditions of analysis, the dependence of the correction factors of these FA on the positions of FA in TAG was minimal and was thus not taken into account. The proportions of FA within TAG were calculated according to the corrected relative abundance of $[RCOO]^-$ ions in the CID spectra. Iterated calculation of all possible FA combinations of TAG within a specific molecular weight species was carried out according to the ACN:DB (*m/z* value of $[M - H]^-$ ion) and the proportions of FA to achieve a result with the lowest error (excess or shortage of FA). On the basis of the results of the iterated calculation, the proportions of the FA were further corrected to reduce the errors introduced during the MS/MS analysis. These corrected relative abundances of FA were then used in the calculation of the regioisomers (11,14).

The differentiation of FA in the primary positions from that in the secondary position of TAG was based on the preferential formation of $[M - H - RCOOH - 100]^-$ ions from FA located in *sn*-1 and *sn*-3 positions (11). For TAG with a FA combination A/B/A, a standard curve can be established of the ratio $[M - H - B - 100]^-/[M - H - A - 100]^-$ (typically ranging from 0.1 to 0.8) with a gradual change in composition of the TAG from 100% *sn*-A-B-A to 100% *sn*-A-A-B + *sn*-B-A-A. Experience has shown that the standard curve depends mainly on the parameters of MS/MS. The influence of the FA with 16 and 18 carbons and 0–3 double bonds was minor, and thus not taken into account in the calculation. The curve was measured and regularly controlled with the TAG pair 1,2-di-(*cis*-9-octadecenoyl)-3-hexadecanoyl-*sn*-glycerol and 1,3-di-(*cis*-9-octadecenoyl)-2-hexadecanoyl-*sn*-glycerol. An experimental equation based on the standard curve, the relative abundance of $[M - H - RCOOH - 100]^-$ and the corrected proportions of the FA were used for differentiating the FA in the secondary position from those in the primary positions of TAG (11–15).

Automatic calculation of data of MS and MS/MS analyses. In the present study, the proportions of ACN:DB species, the proportions of FA combinations, and proportions of regioisomers within each ACN:DB species of TAG were calculated automatically using the software TAGS-100/MSPECTRA (Nutrifen Oy, Naantali, Finland) based on the mathematical principles described previously (14,16).

Statistical analysis. The sea buckthorn samples analyzed in the present study were taken from larger sample groups of sea buckthorn, which were shown by earlier investigations to follow a normal distribution within the subspecies (2,8).

Due to the relatively high deviation in the analysis, the proportions of regioisomers from four repeated analyses were used directly for statistical analysis of the data (n = number of growth sites/varieties × 4 repeats). Some minor regioisomers appeared only in some of the four repeated runs; during the calculation a value of zero was assigned to the missing peaks.

The data analysis was carried out using the statistical program SPSS 11.0 for Windows. One-way ANOVA and independent sample t-test were used in the comparison of the analyzed results of positional distribution of FA in TAG with the random distribution, as well as in the comparison of the molecular weight distribution and regioisomers of TAG among the subspecies. The data from the three subspecies were combined when comparing the analyzed results of positional distribution of FA in TAG with the random distribution. Differences reaching a minimum confidence level of 95% ($P < 0.05$) were defined as being statistically significant.

RESULTS

FA composition of TAG of seeds and berries. The FA compositions of TAG of seeds and berries of three different subspecies are summarized in Table 1. Seed TAG of ssp. *sinensis* contained a higher proportion of oleic acid and a lower proportion of α-linolenic acid compared with those of ssp. *rhamnoides*. In berry TAG, the proportion of palmitic and palmitoleic acids was higher in ssp. *mongolica* than in ssp. *sinensis* and *rhamnoides*. This was accompanied by a lower proportion of oleic acid (4%, vs. 17% in ssp. *sinensis* and *rham-*

TABLE 1
FA Composition of Seeds and Berries of Three Subspecies of Sea Buckthorn (mol%)

Seed TAG	ssp. *sinensis* ($n = 3^a$)	ssp. *rhamnoides* ($n = 3^a$)	ssp. *mongolica* ($n = 3^a$)
FA			
16:0	9.6 ± 0.7	7.1 ± 0.3	8.4 ± 1.3
18:0	2.3 ± 0.4	2.6 ± 0.2	3.1 ± 0.5
18:1n-9	21.3 ± 3.5[a]	16.3 ± 2.3[b]	18.4 ± 4.4[ab]
18:1n-7	2.1 ± 0.3	2.8 ± 0.7	2.2 ± 0.3
18:2n-6	40.1 ± 3.0	39.5 ± 3.1	37.5 ± 3.2
18:3n-3	24.7 ± 3.0[a]	31.7 ± 3.0[b]	30.4 ± 5.2[b]
Berry TAG	ssp. *sinensis* ($n = 4^b$)	ssp. *rhamnoides* ($n = 5^b$)	ssp. *mongolica* ($n = 3^b$)
FA			
16:0	27.1 ± 0.6[a]	23.7 ± 3.2[a]	34.6 ± 1.7[b]
16:1n-7	24.6 ± 2.5[a]	26.8 ± 3.6[a]	34.6 ± 5.4[b]
18:0	1.2 ± 0.2	1.0 ± 0.1	1.1 ± 0.2
18:1n-9	17.4 ± 1.2[a]	17.2 ± 3.0[a]	4.3 ± 1.3[b]
18:1n-7	6.7 ± 0.7	7.7 ± 0.9	6.7 ± 0.8
18:2n-6	14.1 ± 2.0	14.6 ± 3.3	14.3 ± 2.2
18:3n-3	9.0 ± 2.1[a]	8.9 ± 2.1[a]	4.4 ± 0.7[b]

[a]*n* = number of natural growth sites or varieties; ssp. *sinensis*, three natural growth sites in Shanxi, Qinghai, and Gansu, China; ssp. *rhamnoides*, three natural growth sites in Vaasa and Pyhämaa, Finland; ssp. *mongolica*, three commercial varieties from Russia.
[b]*n* = number of natural growth sites or varieties; ssp. *sinensis*, four natural growth sites in Shanxi, Shaanxi, Qinghai, and Gansu, China; ssp. *rhamnoides*, five natural growth sites in Hirsilahti, Siikajoki, Vaasa, and Pyhämaa, Finland; ssp. *mongolica*, three commercial varieties from Russia. [a–b]Means ± SD with different superscripts within a row differ significantly ($P < 0.05$).

noides) and α-linolenic acid (4%, vs. 9% in ssp. *sinensis* and *rhamnoides*) in ssp. *mongolica*.

Molecular weight distribution of seed TAG. The molecular weight distributions of seed TAG of different subspecies of sea buckthorn are summarized in Figure 1. The major ACN species in sea buckthorn seed TAG were 54 (70–80%) and 52 (20–30%) followed by trace amounts of 50 and 56, regardless of the subspecies. Seeds of ssp. *sinensis* contained slightly more palmitic acid, which resulted in a higher proportion of ACN 52 species (27%) and a lower proportion of ACN 54 species (71%) compared with ssp. *mongolica* (21% for ACN 52, 79% for ACN 54) and *rhamnoides* (22% for ACN 52, 78% for ACN 54) ($P < 0.01$ for ACN 52, $P < 0.001$ for ACN 54).

The level of saturation within both the ACN 52 and ACN 54 groups also showed a regular pattern among the three subspecies (Fig. 1). In the ACN 52 group, the proportions of 52:4, 52:3, and 52:2 were significantly higher in TAG of ssp. *sinensis* compared with those of ssp. *mongolica* (9.1% vs. 5.6%, $P < 0.05$; 5.8% vs. 3.0%, $P < 0.05$; and 2.9% vs. 0.9%, $P < 0.01$, respectively). The corresponding values of ssp. *rhamnoides* fell somewhere between the other two subspecies, with higher proportions of 52:4 and 52:2 compared with ssp. *mongolica* ($P < 0.01$; $P < 0.05$), and lower proportions of 52:3 and 52:2 compared with ssp. *sinensis* ($P = 0.05$; $P < 0.05$). The proportion of 52:6 was highest in ssp. *mongolica* and lowest in ssp. *sinensis*; the difference between the two subspecies reached a statistically significant level (4.0% vs. 2.7%, $P = 0.05$).

For the ACN 54 group, the TAG of ssp. *monogolica* seeds contained a significantly higher proportion of 54:9 (6.5%), 54:8 (17.0%), and 54:7 (20.8%) compared with those of ssp. *sinensis* (2.3%, $P < 0.01$; 8.3%, $P < 0.01$; and 14.9%, $P < 0.001$, respectively) and *rhamnoides* (3.9%, $P < 0.01$; 12.5%, $P < 0.1$; and 19.3%, $P < 0.1$, respectively). The proportions of 54:5, 54:4, and 54:3 were highest in ssp. *sinensis* and lowest in ssp. *mongolica* (15.7% vs. 11.2%, $P < 0.05$; 9.1 vs. 3.9%, $P < 0.05$; 3.8% vs. 1.5%, $P < 0.05$, respectively). Ssp. *rhamnoides* again fell between ssp. *sinensis* and *mongolica*, differing from both subspecies in the proportion of 54:8 and 54:7 (Fig. 1). Statistically significant differences were also found between ssp. *rhamnoides* and *mongolica* in the proportions of 54:9 and 54:3 as well as between ssp. *sinensis* and *rhamnoides* in the proportion of 54:4 (Fig. 1).

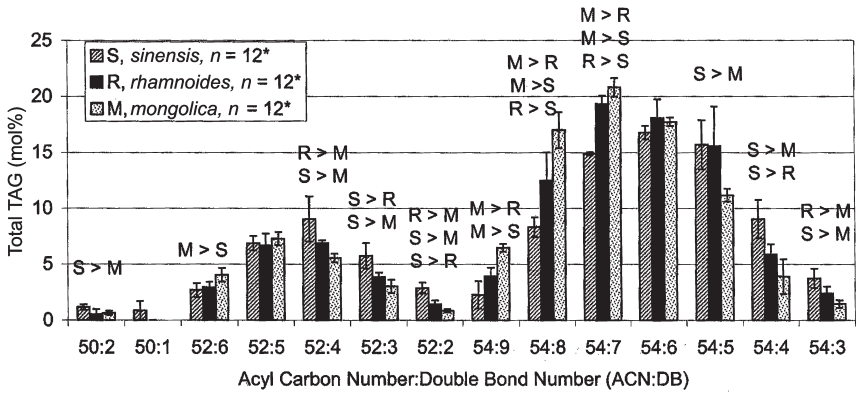

FIG. 1. Molecular weight species of TAG of seeds of three subspecies of sea buckthorn (mol%). Differences among the subspecies are indicated only when the difference reached a statistically significant level ($P < 0.05$). *n = number of natural growth sites (or varieties) × number of repeats (4); ssp. *sinensis*, three natural growth sites in Shanxi, Qinghai, and Gansu, China; ssp. *rhamnoides*, three natural growth sites in Vaasa and Pyhämaa, Finland; ssp. *mongolica*, three commercial varieties from Russia.

Positional distribution of FA in seed TAG. Regioisomers of seven major ACN:DB species 52:3 through 52:5 and 54:5 through 54:8 of seed TAG were analyzed by MS/MS. The results are summarized in Table 2.

ACN:DB 52:3. The FA combination 16:0/18:2/18:1 constituted on average 95% of the ACN:DB group 52:3, the proportion being higher in ssp. *rhamnoides* than in ssp. *mongolica* (98.0% vs. 92.4%, $P < 0.05$). Due to the low content of stearic acid and palmitoleic acid in sea buckthorn seed oil, TAG with FA combinations 16:0/18:3/18:0, 16:1/18:2/18:0, and 16:1/18:1/18:1 existed in trace amounts only.

Linoleic acid (18:2n-6) was mainly in the *sn*-2 position, whereas palmitic acid (16:0) was preferentially located in the *sn*-1/3 positions. FA of 18:1 did not show a clear preference between the primary and secondary positions.

Comparing the three subspecies (Table 2), the proportion of 1(3)-palmitoyl-2-oleoyl/vaccenoyl-3(1)-linoleoyl-*sn*-glycerols (*sn*-16:0-18:1-18:2 + *sn*-18:2-18:1-16:0) in the 52:3 group was higher in ssp. *mongolica* than in ssp. *rhamnoides* (48.9% vs. 30.1%, $P < 0.05$). This difference was accompanied by a higher proportion of TAG with 18:2 in the *sn*-2 position (*sn*-16:0-18:2-18:1 + *sn*-18:1-18:2-16:0) in ssp. *rhamnoides* compared with ssp. *mongolica* (64.4% vs. 39.6%, $P < 0.05$) and *sinensis* (64.4% vs. 56.5%, $P = 0.05$). In addition, differences among the subspecies were also recognized in some minor regioisomers. The proportion of 1(3)-palmitoyl-2-stearyl-3(1)-linolenoyl-*sn*-glycerols (*sn*-16:0-18:0-18:3 + *sn*-18:3-18:0-16:0) was higher in ssp. *mongolica* compared with ssp. *rhamnoides* (2.6% vs. 0.2%, $P < 0.01$) and *sinensis* (2.6% vs. 0.9%, $P < 0.05$); the proportion of 1(3)-palmitoleoyl-2-oleoyl/vaccenoyl-3(1)-oleoyl/vaccenoyl-*sn*-glycerols (*sn*-16:1-18:1-18:1 + *sn*-18:1-18:1-16:1) was highest in ssp. *sinensis* (1.8% vs 0.2% and 0.3%, $P < 0.05$).

ACN:DB 52:4. Two major FA combinations, 16:0/18:3/18:1 and 16:0/18:2/18:2, together accounted for 96–97% of 52:4 TAG.

Within the combination 16:0/18:3/18:1, 18:1 FA and α-linolenic acid ($P = 0.07$) were preferentially located in the secondary position, whereas palmitic acid was mainly found in the primary positions, differing significantly from random distribution. In the FA combination 16:0/18:2/18:2, 1(3)-palmitoyl-2-linoleoyl-3(1)-linoleoyl-*sn*-glycerols (*sn*-16:0-18:2-18:2 + *sn*-18:2-18:2-16:0) accounted for 72%, 80%, and 87%, respectively, in the three subspecies, showing a clear dominance of linoleic acid in the *sn*-2 position.

Ssp. *rhamnoides* differed from ssp. *mongolica* by having higher proportions of 1(3)-palmitoyl-2-linolenoyl-3(1)-oleoyl/vaccenoyl-*sn*-glycerols (*sn*-16:0-18:3-18:1 + *sn*-18:1-18:3-16:0, 22.5% vs. 12.6%, $P < 0.05$) and 1-linoleoyl-2-palmitoyl-3-linoleoyl-*sn*-glycerol (*sn*-18:2-16:0-18:2, 15.9% vs. 7.0%, $P < 0.05$) and a lower proportion of 1(3)-palmitoyl-2-oleoyl/vaccenoyl-3(1)-linolenoyl-*sn*-glycerols (*sn*-16:0-18:1-18:3 + *sn*-18:3-18:1-16:0, 16.0% vs. 28.3%, $P < 0.05$).

ACN:DB 52:5. The most abundant FA combination of the 52:5 group was 16:0/18:3/18:2, accounting for an average of 93% of 52:5 TAG of the samples analyzed (Table 2).

Ssp. *sinensis* had a higher proportion of the combination 16:1/18:2/18:2 (6.3% vs. 4.8%, $P < 0.05$) and a lower proportion of the combination 16:0/18:3/18:2 (92.5% vs. 94.5%, $P < 0.01$) than ssp. *mongolica*. The proportion of the combination 16:0/18:3/18:2 was also lower in ssp. *rhamnoides* compared with the value in ssp. *mongolica* (91.7% vs. 94.5%, $P < 0.05$).

Within the most abundant FA combination, 16:0/18:3/18:2, 18:2 was the preferred FA in the *sn*-2 position. The FA 16:0 and 18:3 were mainly located in the primary (*sn*-1/3) positions.

Within the FA combination 16:1/18:2/18:2, ssp. *sinensis* had a higher proportion of 1(3)-palmitoleoyl-2-linoleoyl-3(1)-linoleoyl-*sn*-glycerols (*sn*-16:1-18:2-18:2 + *sn*-18:2-18:2-16:1) than ssp. *mongolica* (5.6% vs. 3.2%, $P < 0.05$).

ACN:DB 54:5. The 54:5 TAG group consisted of three different FA combinations: 18:2/18:2/18:1, 18:3/18:1/18:1, and 18:3/18:2/18:0. Within each of the FA combinations, the distribution of the FA between primary and secondary positions did not differ significantly from random distribution (Table 2).

Ssp. *mongolica* had a lower proportion of the FA combination 18:2/18:2/18:1 compared with ssp. *sinensis* (55.5 % vs. 61.7% within 54:5, $P = 0.06$) and *rhamnoides* (55.5% vs. 65.0% within 54:5, $P < 0.001$). This was accompanied by a higher proportion of the FA combination 18:3/18:2/18:0 (18.3% in ssp. *mongolica* vs. 11.5% in *rhamnoides,* $P < 0.01$, and vs. 6.1% in ssp. *sinensis*, $P < 0.001$). The differences between ssp. *sinensis* and *rhamnoides* in the proportion of the FA combinations 18:3/18:2/18:0 (6.1% vs. 11.5%) and 18:3/18:1/18:1 (32.1% vs. 23.4%) were also statistically significant ($P < 0.05$).

Differences were also found among the subspecies in the proportion of 1(3)-linolenoyl-2-oleoyl/vaccenoyl-3(1)-oleoyl/vaccenoyl-*sn*-glycerols (*sn*-18:3-18:1-18:1 + *sn*-18:1-18:1-18:3; *sinensis* > *rhamnoides*, $P < 0.05$) and 1(3)-linoleoyl-2-linoleoyl-3(1)-oleoyl/vaccenoyl-*sn*-glycerols (*sn*-18:2-18:2-18:1 + *sn*-18:1-18:2-18:2; *rhamnoides* > *mongolica*, $P < 0.05$).

ACN:DB 54:6. The dominating FA combinations of this group were 18:3/18:2/18:1 and 18:2/18:2/18:2. In addition, 18:3/18:3/18:0 was present in trace amount in some of the samples analyzed (Table 2).

Within the FA combination 18:3/18:2/18:1, 18:1 clearly dominated in the *sn*-2 position, whereas 18:2 was mostly found in the primary positions. The FA 18:3 did not discriminate between the primary and secondary positions.

The proportion of TAG with the FA combination 18:3/18:3/18:0 was higher in ssp. *rhamnoides* than in ssp. *sinensis* (2.2% vs. 0.4%, $P < 0.05$). A difference was seen in the proportion of 1(3)-oleoyl/vaccenoyl-2-linoleoyl-3(1)-linolenoyl-*sn*-glycerols (*sn*-18:3-18:2-18:1 + *sn*-18:1-18:2-18:3) between ssp. *sinensis* and *mongolica* (11.5% vs. 1.1%, $P < 0.05$).

ACN:DB 54:7. ACN:DB 54:7 was the most abundant TAG group in the seed of ssp. *rhamnoides* (19.3% of seed TAG) and *mongolica* (20.8% of seed TAG). Of the two FA combinations in this TAG group, 18:3/18:2/18:2 dominated over

TABLE 2
Positional Distribution of FA in Major Molecular Weight Species of TAG of Seeds of Sea Buckthorn of Different Subspecies[a]

	ssp. *sinensis* ($n = 12^b$)	ssp. *rhamnoides* ($n = 12^b$)	ssp. *mongolica* ($n = 12^b$)
52:3	**5.8 ± 1.1[a]**	**3.8 ± 0.4[b]**	**3.0 ± 0.6[b]**
16:1/18:2/18:0	0.4 ± 0.6	0.4 ± 0.7	0.6 ± 0.9
sn-16:1-18:2-18:0 + *sn*-18:0-18:2-16:1	0.4 ± 0.6	0 ± 0	0.5 ± 0.8
sn-18:2-16:1-18:0 + *sn*-18:0-16:1-18:2	0 ± 0	0.4 ± 0.7	0.2 ± 0.3
sn-18:2-18:0-16:1 + *sn*-16:1-18:0-18:2	nd	nd	nd
16:1/18:1/18:1	2.0 ± 0.6	0.3 ± 0.4	0.3 ± 0.5
sn-16:1-18:1-18:1 + *sn*-18:1-18:1-16:1	1.8 ± 0.2[a]	0.2 ± 0.1[b]	0.3 ± 0.5[b]
sn-18:1-16:1-18:1	0.3 ± 0.5	0.2 ± 0.3	0 ± 0
16:0/18:3/18:0	3.4 ± 3.2	1.3 ± 1.2	6.6 ± 1.8
sn-16:0-18:3-18:0 + *sn*-18:0-18:3-16:0	1.4 ± 2.1	0.9 ± 1.2	2.8 ± 2.1
sn-18:3-16:0-18:0 + *sn*-18:0-16:0-18:3	1.0 ± 0.9	0.2 ± 0.3	1.2 ± 1.0
sn-16:0-18:0-18:3 + *sn*-18:3-18:0-16:0	0.9 ± 0.8[a]	0.2 ± 0.3[a]	2.6 ± 0.6[b]
16:0/18:2/18:1	94.2 ± 2.7[ab]	98.0 ± 2.0[a]	92.4 ± 0.9[b]
sn-16:0-18:2-18:1 + *sn*-18:1-18:2-16:0*	56.5 ± 4.8[a]	64.4 ± 2.1[b]	39.6 ± 13.7[a]
sn-18:2-16:0:18:1 + *sn*-18:1-16:0-18:2*	4.6 ± 6.7	3.5 ± 2.1	4.0 ± 3.2
sn-16:0-18:1-18:2 + *sn*-18:2-18:1-16:0	33.1 ± 7.3[ab]	30.1 ± 1.0[a]	48.9 ± 10.6[b]
52:4	**9.1 ± 2.0[a]**	**6.9 ± 0.3[a]**	**5.6 ± 0.4[b]**
16:1/18:2/18:1	3.5 ± 2.1	2.7 ± 2.0	4.1 ± 0.9
sn-16:1-18:2-18:1 + *sn*-18:1-18:2-16:1	0.3 ± 0.3	0.2 ± 0.8	1.2 ± 2.1
sn-16:1-18:1-18:2 + *sn*-18:2-18:1-16:1	2.6 ± 1.5	2.0 ± 1.3	2.6 ± 1.7
sn-18:2-16:1-18:1 + *sn*-18:1-16:1-18:2	0.6 ± 1.0	0.5 ± 0.9	0.4 ± 0.8
16:0/18:3/18:1	45.0 ± 12.6	39.3 ± 5.5	41.3 ± 2.0
sn-16:0-18:3-18:1 + *sn*-18:1-18:3-16:0	18.2 ± 12.5[ab]	22.5 ± 5.7[a]	12.6 ± 0.3[b]
sn-18:3-16:0-18:1 + *sn*-18:1-16:0-18:3*	1.2 ± 1.7	0.9 ± 0.6	0.4 ± 0.6
sn-16:0-18:1-18:3 + *sn*-18:3-18:1-16:0*	25.6 ± 2.4[ab]	16.0 ± 7.7[a]	28.3 ± 0.9[b]
16:0/18:2/18:2	51.5 ± 14.7	57.9 ± 5.4	54.6 ± 1.2
sn-18:2-16:0-18:2*	10.1 ± 9.0[ab]	15.9 ± 8.1[a]	7.0 ± 3.4[b]
sn-16:0-18:2-18:2 + *sn*-18:2-18:2-16:0**	41.3 ± 22.7	41.9 ± 8.7	47.6 ± 3.1
52:5	**6.9 ± 0.6**	**6.7 ± 1.0**	**7.3 ± 0.6**
16:1/18:3/18:1	1.2 ± 2.3	2.7 ± 3.1	0.7 ± 1.3
sn-16:1-18:3-18:1 + *sn*-18:1-18:3-16:1	0.3 ± 0.7	0.2 ± 0.6	0.1 ± 0.4
sn-16:1-18:1-18:3 + *sn*-18:3-18:1-16:1	0.6 ± 1.4	1.5 ± 2.1	0.3 ± 0.7
sn-18:3-16:1-18:1 + *sn*-18:1-16:1-18:3	0.2 ± 0.8	1.1 ± 1.7	0.3 ± 0.6
16:1/18:2/18:2	6.3 ± 2.1[a]	5.6 ± 2.8[ab]	4.8 ± 1.6[b]
sn-16:1-18:2-18:2 + *sn*-18:2-18:2-16:1	5.6 ± 2.0[a]	4.2 ± 2.6[ab]	3.2 ± 1.9[b]
sn-18:2-16:1-18:2	0.7 ± 1.6	1.4 ± 2.1	1.6 ± 2.3
16:0/18:3/18:2	92.5 ± 2.0[a]	91.7 ± 3.3[a]	94.5 ± 1.5[b]
sn-16:0-18:2-18:3 + *sn*-18:3-18:2-16:0*	58.8 ± 10.0	56.1 ± 11.4	63.2 ± 11.4
sn-16:0-18:3-18:2 + *sn*-18:2-18:3-16:0*	21.2 ± 15.1	17.4 ± 12.9	15.5 ± 11.1
sn-18:3-16:0-18:2 + *sn*-18:2-16:0-18:3*	12.6 ± 10.6	18.2 ± 12.4	15.8 ± 3.5
54:5	**15.7 ± 2.2[a]**	**15.6 ± 3.6[ab]**	**11.2 ± 0.6[b]**
18:3/18:2/18:0	6.1 ± 3.9[a]	11.5 ± 5.9[b]	18.3 ± 2.4[c]
sn-18:3-18:2-18:0 + *sn*-18:0-18:2-18:3	2.2 ± 2.7	3.1 ± 4.9	6.4 ± 6.9
sn-18:2-18:3-18:0 + *sn*-18:0-18:3-18:2	2.6 ± 3.0	3.9 ± 5.5	7.3 ± 6.4
sn-18:3-18:0-18:2 + *sn*-18:2-18:0-18:3	1.3 ± 3.2	4.6 ± 5.8	4.6 ± 4.6
18:3/18:1/18:1	32.1 ± 9.8[a]	23.4 ± 6.3[b]	26.2 ± 5.0[ab]
sn-18:3-18:1-18:1 + *sn*-18:1-18:1-18:3	25.4 ± 14.3[a]	12.3 ± 12.1[b]	17.8 ± 9.2[ab]
sn-18:1-18:3-18:1	6.7 ± 7.8	11.1 ± 10.2	8.4 ± 8.8
18:2/18:2/18:1	61.7 ± 8.8[a]	65.0 ± 4.2[a]	55.5 ± 3.6[b]
sn-18:2-18:2-18:1 + *sn*-18:1-18:2-18:2	40.3 ± 15.1[ab]	48.6 ± 17.4[a]	32.7 ± 13.9[b]
sn-18:2-18:1-18:2	21.4 ± 15.1	16.4 ± 14.2	22.8 ± 13.8
54:6	**16.8 ± 0.6**	**18.1 ± 1.7**	**17.7 ± 0.4**
18:3/18:3/18:0	0.4 ± 1.0[a]	2.2 ± 2.6[b]	2.6 ± 3.3[ab]
sn-18:0-18:3-18:3 + *sn*-18:3-18:3-18:0	0.4 ± 1.0	1.9 ± 2.4	2.1 ± 2.6
sn-18:3-18:0-18:3	0 ± 0	0.3 ± 0.5	0.5 ± 1.0

(continued)

TABLE 2 (continued)

18:3/18:2/18:1	83.0 ± 15.3	87.5 ± 9.7	87.0 ± 3.4
sn-18:3-18:2-18:1 + *sn*-18:1-18:2-18:3*	11.5 ± 15.2[a]	8.8 ± 14.3[ab]	1.1 ± 2.6[b]
sn-18:2-18:3-18:1 + *sn*-18:1-18:3-18:2	26.8 ± 15.5	28.0 ± 19.2	35.0 ± 8.3
sn-18:3-18:1-18:2 + *sn*-18:2-18:1-18:3*	44.8 ± 21.3	50.7 ± 15.1	50.9 ± 8.0
18:2/18:2/18:2	16.6 ± 15.7	10.3 ± 9.4	10.4 ± 4.2
54:7	**14.9 ± 0.1[a]**	**19.3 ± 0.7[b]**	**20.8 ± 0.8[b]**
18:3/18:3/18:1	31.5 ± 10.0	29.7 ± 1.9	30.5 ± 4.0
sn-18:3-18:3-18:1 + *sn*-18:1-18:3-18:3*	11.1 ± 5.0	12.3 ± 6.3	14.2 ± 5.5
sn-18:3-18:1-18:3*	20.4 ± 10.4	17.4 ± 7.4	16.4 ± 7.5
18:3/18:2/18:2	68.4 ± 10.0	70.3 ± 1.9	69.4 ± 3.9
sn-18:3-18:2-18:2 + *sn*-18:2-18:2-18:3*	50.5 ± 11.7	52.5 ± 13.2	58.6 ± 13.1
sn-18:2-18:3-18:2*	18.0 ± 4.1[a]	17.8 ± 12.5[ab]	10.9 ± 9.9[b]
54:8	**8.3 ± 0.9[a]**	**12.5 ± 2.5[ab]**	**17.0 ± 1.6[b]**
18:3/18:3/18:2	100	100	100
sn-18:3-18:3-18:2 + *sn*-18:2-18:3-18:3*	45.7 ± 10.8[a]	37.0 ± 6.7[b]	44.3 ± 9.4[a]
sn-18:3-18:2-18:3*	54.3 ± 10.8[a]	63.0 ± 6.7[b]	55.7 ± 9.4[a]
TAG with *sn*-2 18:1	22.5 ± 6.8	19.5 ± 1.0	20.1 ± 0.5
TAG with *sn*-2 18:2	34.6 ± 8.7	38.7 ± 4.3	36.8 ± 2.0
TAG with *sn*-2 18:3	17.3 ± 1.8[a]	21.2 ± 2.8[ab]	23.0 ± 1.9[b]

[a]Values in bold are molar percentage of total TAG; other values represent molar percentages within each acyl carbon number:double bond (ACN:DB) group. [b]*n* = number of natural growth sites (or varieties) × number of repeats (4); ssp. *sinensis*, three natural growth sites in Shanxi, Qinghai, and Gansu, China; ssp. *rhamnoides*, three natural growth sites in Vaasa and Pyhämaa, Finland; ssp. *mongolica*, three commercial varieties from Russia. [a–c]Means ± SD with different superscripts within a row differ significantly ($P < 0.05$). Asterisks indicate statistically significant difference from random system, $*P < 0.001$; $**P < 0.05$.

18:3/18:3/18:1 (69.4% vs. 30.6% of 54:7 TAG). In the former combination, linoleic acid was mainly found in the *sn*-2 position (Table 2). α-Linolenic acid, on the contrary, preferred the primary (*sn*-1/3) positions. This was shown as an overall ratio of 3.5:1 between the two types of regioisomers in the samples analyzed.

In the FA combination18:3/18:3/18:1, oleic/vaccenic acids were the preferred FA in the *sn*-2 position (Table 2).

When comparing the three subspecies, the only statistically significant difference found was a higher proportion of 1-linoleoyl-2-linolenoyl-3-linoleoyl-*sn*-glycerols in ssp. *sinensis* than in ssp. *mongolica* (18.0% vs. 10.9% of 54:7, $P < 0.05$).

ACN:DB 54:8. In the seeds of ssp. *sinensis,* ACN:DB 54:8 species (8.3% of seed TAG) accounted for less than half of that of ssp. *mongolica* (17.0%). The proportion in ssp. *rhamnoides* (12.5 % of seed TAG) fell between the other two subspecies. The only FA combination in this group was 18:3/18:3/18:2. Linoleic acid was preferred in the *sn*-2 position (Table 2).

Ssp. *rhamnoides* differed from the other two subspecies in its higher proportion of *sn*-18:3-18:2-18:3 ($P < 0.05$) and its lower proportion of 1(3)-linoleoyl-2-linolenoyl-3(1)-linolenoyl-*sn*-glycerols (*sn*-18:2-18:3-18:3 + *sn*-18:3-18:3-18:2, $P < 0.05$).

Molecular weight distribution of TAG of whole berries. Figure 2 presents the molecular weight distribution of the TAG of whole berries (A) and fruit pulp/peel (B) of different subspecies. The major ACN of TAG from the whole berries were 48, 50, 52, and 54. Ssp. *sinensis* and *rhamnoides* showed similar patterns of molecular weight distribution of TAG, which clearly differed from that of ssp. *mongolica*. Overall, ssp. *mongolica* had a higher proportion of TAG with 48 acyl carbons (47.6% in ssp. *mongolica* vs 22.1% in ssp. *sinensis* and 23.3% in ssp. *rhamnoides*), whereas ssp. *sinensis* and *rhamnoides* were more abundant in TAG with ACN 52 (21.6% and 22.4% vs. 10.2%) and 54 (20.1% and 17.4% vs. 5.8%). Statistically significant differences among the subspecies were found in the proportions of most of the molecular weight species detected (Fig. 2A).

Comparing the molecular weight distribution of TAG from whole berries (Fig. 2A) and fruit flesh/peel (Fig. 2B), it is clear that the TAG of 54 acyl carbons were mostly from the seeds. Seed content is known to be lower in berries of ssp. *mongolica* than in berries of ssp. *sinensis* and *rhamnoides* (2,17). Thus, the difference observed in the molecular weight distribution of TAG among the subspecies was partially due to the different seed content in the berries. In addition, the differences in molecular weight species also reflected variations in overall FA composition of TAG (2,8,17) as well as FA combination in TAG molecules among the subspecies.

The major ACN:DB species of TAG of the whole berries (i.e., mainly of fruit flesh and peel) were 48:1, 48:2, 50:2, and 50:3, followed by 52:2, 52:3, and 52:4. TAG of ACN:DB species 48:1, 48:2, 50:2, and 50:3 constituted together up to 46%, 49%, and 71% of berry TAG of ssp. *sinensis*, *rhamnoides*, and *mongolica,* respectively.

Positional distribution of FA in berry TAG. Positional distributions of FA of the four most abundant ACN:DB species (48:1, 48:2, 50:2, and 50:3) were analyzed in berry TAG because these were the most important molecular weight species of TAG from the fruit pulp/peel. The results are summarized in Table 3.

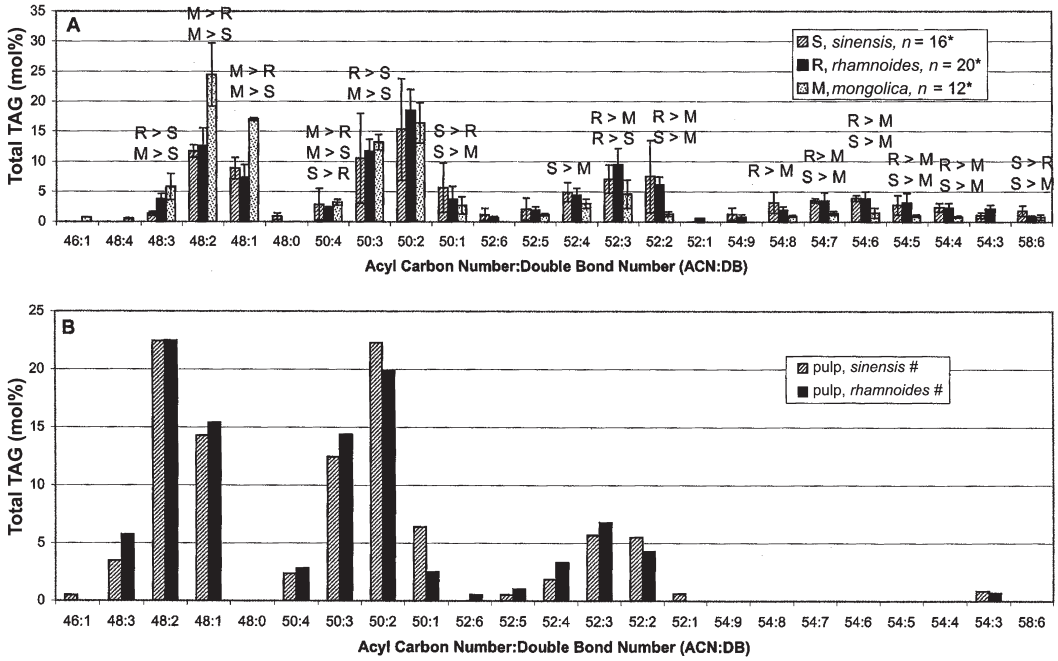

FIG 2. Molecular weight species of TAG of (A) whole berries of three subspecies and (B) fruit flesh/peel of two subspecies of sea buckthorn (mol%). Differences among the subspecies are indicated only when the difference reached a statistically significant level ($P < 0.05$). *for whole berries, n = number of natural growth sites (or varieties) × number of repeats (4); ssp. *sinensis,* four natural growth sites in Shanxi, Shaanxi, Qinghai, and Gansu, China; ssp. *rhamnoides,* five natural growth sites in Hirsilahti, Siikajoki, Vaasa, and Pyhämaa, Finland; ssp. *mongolica,* three commercial varieties from Russia. #ssp. *sinensis,* average of four analyses of one sample from a natural growth site in Shanxi, China; ssp. *rhamnoides,* average of four analyses of one sample from a natural growth site in Pyhämaa, Finland.

ACN:DB 48:1. This group consisted of TAG of only one FA combination, 16:1/16:0/16:0 (Table 3). The dominating regioisomer was 1-palmitoyl-2-palmitoleoyl-3-palmitoyl-*sn*-glycerol (*sn*-16:0-16:1-16:0), representing 82–87% of TAG of ACN:DB 48:1 in the three subspecies. Palmitoleic acid (16:1n-7) was the preferred FA in the *sn*-2 position, whereas 16:0 was favored in the *sn*-1/3 positions.

ACN:DB 48:2. This group consisted of only TAG with the FA combination 16:1/16:1/16:0. Molecular species with palmitoleic acid in the *sn*-2 position (*sn*-16:1-16:1-16:0 + *sn*-16:0-16:1-16:1) were by far the major regioisomers of this group (Table 3). The minor molecular species with palmitic acid in the *sn*-2 position represented less than 10% of the TAG of this ACN:DB group.

Compared with ssp. *sinensis*, ssp. *mongolica* had a higher proportion of regioisomers with 16:1 in the *sn*-2 position (98.4% vs. 86.1%, $P < 0.05$) and a lower proportion of regioisomers with 16:0 in the *sn*-2 position (1.6% vs. 13.9%, $P < 0.05$). The corresponding values of ssp. *rhamnoides* fell between the other two subspecies, almost statistically different ($P = 0.07$) from those of ssp. *mongolica*.

ACN:DB 50:2. Two major FA combinations, 16:1/16:0/18:1 (92.8%, 92.6%, and 63.6% of 50:2, respectively, in the three subspecies) and 16:0/16:0/18:2 (6.3%, 6.5%, and 34.6% of 50:2, respectively, in the three subspecies), were identified in this ACN:DB group. In addition, traces of TAG with the FA combination 16:1/16:1/18:0 were present (about 1% of 50:2 on average).

Of the three subspecies, ssp. *mongolica* had the lowest proportion of TAG of the FA combination 16:1/16:0/18:1 (92.8% and 92.6% in ssp. *sinensis* and *rhamnoides*, respectively, vs. 63.6% in ssp. *mongolica*, $P < 0.001$) and the highest proportion of the combination 16:0/16:0/18:2 (34.6% in ssp. *mongolica* vs. 6.3% in ssp. *sinensis* and 6.5% in ssp. *rhamnoides*, $P < 0.001$) within the group 50:2. The proportion of 1(3)-palmitoyl-2-palmitoleoyl-3(1)-oleoyl/vaccenoyl-*sn*-glycerols (*sn*-16:0-16:1-18:1 + *sn*-18:1-16:1-16:0) was highest in ssp. *rhamnoides* (46.0% vs. 22.5% in *sinensis,* $P < 0.01$; 46.0% vs. 31.1% in ssp. *mongolica*, $P = 0.05$). The highest proportion of 1(3)-palmitoleoyl-2-oleoyl/vaccenoyl-3(1)-palmitoyl-*sn*-glycerols (*sn*-16:1-18:1-16:0 + *sn*-16:0-18:1-16:1) was found in ssp. *sinensis* (62.2% vs. 41.7%, $P < 0.05$ compared with ssp. *rhamnoides*; 62.2% vs. 25.0%, $P < 0.001$ compared with ssp. *mongolica*).

Being more abundant in TAG with the FA combination 16:0/16:0/18:2, ssp. *mongolica* contained higher proportions of both types of regioisomers within this combination, compared with the other two subspecies ($P < 0.001$) (Table 3).

ACN:DB 50:3. This group consisted of TAG of two FA combinations, 16:1/16:1/18:1 and 16:1/16:0/18:2. Within the combination 16:1/16:0/18:2, linoleic acid was favored in the *sn*-2 position, whereas palmitic acid was mainly located in the *sn*-1/3 po-

TABLE 3
Positional Distribution of FA in Major Molecular Weight Species of TAG of Berries of Sea Buckthorn of Different Subspecies[a]

	ssp. *sinensis* ($n = 12^b$)	ssp. *rhamnoides* ($n = 12^b$)	ssp. *mongolica* ($n = 12^b$)
48:1	**9.4 ± 1.9[a]**	**7.2 ± 2.0[a]**	**17.1 ± 0.3[b]**
16:1/16:0/16:0	100	100	100
sn-16:1-16:0-16:0 + *sn*-16:0-16:0-16:1*	17.7 ± 4.4	13.1 ± 3.7	15.0 ± 3.8
sn-16:0-16:1-16:0*	82.3 ± 4.4	86.9 ± 3.7	85.0 ± 3.8
48:2	**11.4 ± 0.9[a]**	**12.4 ± 2.9[a]**	**24.6 ± 5.4[b]**
16:1/16:1/16:0	100	100	100
sn-16:1-16:1-16:0 + *sn*-16:0-16:1-16:1*	86.1 ± 4.6[a]	92.1 ± 3.1[ab]	98.4 ±1.5[b]
sn-16:1-16:0-16:1*	13.9 ± 4.6[a]	7.9 ± 3.1[ab]	1.6 ± 1.5[b]
50:2	**18.2 ± 2.6**	**18.2 ± 3.3**	**16.5 ± 3.3**
16:1/16:1/18:0	0.8 ± 1.6	0.9 ± 1.9	1.6 ± 2.8
sn-16:1-16:1-18:0 + *sn*-18:0-16:1-16:1	0.8 ± 1.6	0.7 ± 1.5	1.5 ± 2.5
sn-16:1-18:0-16:1	0.0 ± 0.0	0.2 ± 0.8	0.2 ± 0.5
16:1/16:0/18:1	92.8 ± 6.8[a]	92.6 ± 6.3[a]	63.6 ± 12.3[b]
sn-16:1-16:0-18:1 + *sn*-18:1-16:0-16:1*	8.2 ± 11.5	4.9 ± 9.6	7.5 ± 9.8
sn-16:0-16:1:18:1 + *sn*-18:1-16:1-16:0	22.5 ± 21.3[b]	46.0 ± 17.3[a]	31.1 ± 18.3[b]
sn-16:1-18:1-16:0 + *sn*-16:0-18:1-16:1*	62.2 ± 21.8[a]	41.7 ± 18.4[b]	25.0 ± 14.0[c]
16:0/16:0/18:2	6.3 ± 6.8[a]	6.5 ± 6.9[a]	34.6 ± 12.8[b]
sn-16:0-16:0-18:2 + *sn*-18:2-16:0-16:0*	1.0 ± 1.5[a]	2.5 ± 3.8[ab]	6.0 ± 7.2[b]
sn-16:0-18:2-16:0*	5.3 ± 6.4[a]	3.9 ± 5.5[a]	28.6 ± 15.3[b]
50:3	**6.5 ± 1.0[a]**	**11.5 ± 1.9[b]**	**13.2 ± 1.2[b]**
16:1/16:1/18:1	60.9 ± 24.9[a]	55.5 ± 15.3[a]	25.0 ± 4.1[b]
sn-16:1-16:1-18:1 + *sn*-18:1-16:1-16:1	31.3 ± 30.6[ab]	39.2 ± 14.8[a]	18.5 ± 8.2[b]
sn-16:1-18:1-16:1	29.6 ± 31.6[a]	16.3 ± 20.8[ab]	6.5 ± 8.0[b]
16:1/16:0/18:2	39.1 ± 23.8[a]	44.5 ± 15.3[a]	75.0 ± 4.1[b]
sn-16:1-16:0-18:2 + *sn*-18:2-16:0-16:1*	3.2 ± 6.7	8.2 ± 14.4	6.0 ± 16.7
sn-16:0-16:1-18:2 + *sn*-18:2-16:1-16:0	17.4 ± 12.0[ab]	9.9 ± 13.7[a]	33.1 ± 20.7[b]
sn-16:1-18:2-16:0 + *sn*-16:0-18:2-16:1*	18.5 ± 21.1	26.5 ± 22.1	35.9 ± 17.4
TAG with *sn*-2 16:1	**24.5 ± 4.7[a]**	**31.6 ± 3.8[b]**	**50.8 ± 4.5[c]**
TAG with *sn*-2 18:1	**13.2 ± 3.9[a]**	**9.5 ± 4.1[b]**	**5.0 ± 2.4[c]**
TAG with *sn*-2 18:2	**2.2 ± 1.4[a]**	**3.8 ± 3.0[a]**	**9.5 ± 3.1[b]**

[a]Values in bold are molar percentage of total TAG; other values represent molar percentages within each ACN:DB group. [b]*n* = number of natural growth sites (or varieties) × number of repeats (4); ssp. *sinensis*, three natural growth sites in Shanxi, Qinghai, and Gansu, China; ssp. *rhamnoides*, three natural growth sites in Vaasa and Pyhämaa, Finland; ssp. *mongolica*, three commercial varieties from Russia. [a–c]Means ± SD with different superscripts within a row differ significantly ($P < 0.05$) *Asterisk indicates statistically significant difference from random system, $P < 0.001$.

sitions ($P < 0.001$ compared with random distribution). Palmitoleic acid did not show a clear discrimination between primary and secondary positions. This was shown as a higher average proportion of 1(3)-palmitoleoyl-2-linoleoyl-3(1)-palmitoyl-*sn*-glycerols (*sn*-16:1-18:2-16:0 + *sn*-16:0-18:2-16:1, 27.0% of 50:3) and 1(3)-palmitoyl-2-palmitoleoyl-3(1)-linoleoyl-*sn*-glycerols (*sn*-16:0-16:1-18:2 + *sn*-18:2-16:1-16:0, 20.1% of 50:3) than of 1(3)-palmitoleoyl-2-palmitoyl-3(1)-linoleoyl-*sn*-glycerols (*sn*-16:1-16:0-18:2 + *sn*-18:2-16:0-16:1, 5.8% of 50:3) ($P < 0.001$). It is worth noticing the high deviation in the analysis results for this group.

Among the three subspecies, ssp. *mongolica* had the lowest proportion of TAG of the FA combination 16:1/16:1/18:1 (25.0% vs. 60.9% in ssp. *sinensis* and 55.5% in ssp. *rhamnoides*, $P < 0.001$) and the highest proportion of those with the FA combination 16:1/16:0/18:2 (75.0% vs. 39.1% and 44.5% in ssp. *sinensis* and *rhamnoides*, respectively, $P < 0.001$) (Table 3). Concerning the regioisomers, ssp. *mongolica* was richer in 1(3)-palmitoyl-2-palmitoleoyl-3(1)-linoleoyl-*sn*-glycerols (*sn*-16:0-16:1-18:2 + *sn*-18:2-16:1-16:0, 33.1% vs. 9.9%, $P < 0.01$) and poorer in 1(3)-palmitoleoyl-2-palmitoleoyl-3(1)-oleoyl/vaccenoyl-*sn*-glycerols (*sn*-16:1-16:1-18:1 + *sn*-18:1-16:1-16:1, 18.5% vs. 39.2%, $P < 0.01$) compared with ssp. *rhamnoides*. Differences were also found between ssp. *mongolica* and *sinensis*, the latter being richer in *sn*-16:1-18:1-16:1 (6.5% vs. 29.6%, $P < 0.05$).

TAG with sn-2 unsaturated FA. On average TAG with linoleic acid in the *sn*-2 position represented about 37%, and those with *sn*-2 oleic/vaccenic acid and α-linolenic acid about 21% each, of the molecular weight species of seed TAG analyzed (Table 2). The proportion of TAG with α-linolenic acid in the *sn*-2 position was higher in seed TAG of ssp. *mongolica* than in those of ssp. *sinensis* (23.0% vs. 17.3%, $P < 0.05$).

The TAG with palmitoleic (50.8% vs. 24.5% in ssp. *sinensis* and 31.6% in ssp. *rhamnoides*, $P < 0.01$ among three subspecies) and linoleic (9.5% vs. 2.2% in ssp. *sinensis* and 3.8%

in ssp. *rhamnoides*, $P < 0.001$) acids in the *sn*-2 position represented a higher proportion of berry TAG of ssp. *mongolica* compared with the other two subspecies. The situation was reversed in the case of TAG with oleic/vaccenic acid in the *sn*-2 position (5.0% in ssp. *mongolica* vs. 13.2% in ssp. *sinensis* and 9.5% in ssp. *rhamnoides*, $P < 0.01$) (Table 3).

DISCUSSION

The research group in our department has earlier reported the molecular weight distribution and FA combinations of supercritical CO_2-extracted seed oil and pulp oil of sea buckthorn (18), as well as molecular weight species of seed oil of wild sea buckthorn in Finland (19). The former was a qualitative analysis by capillary supercritical fluid chromatography-atmospheric pressure chemical ionization mass spectrometry, and the latter a quantitative analysis using chemical ionization mass spectrometry. The reported results were in agreement with the findings of the present study.

Using an enzymatic method, Ozerinina *et al.* investigated the TAG of seeds of three different cultivars of *H. rhamnoides* ssp. *mongolica*, Dar Katuni, Maslichnaya, and Shcherbinka-1 (20) and the mesocarp of four different origins (21). The FA of the primary and secondary positions were differentiated. TAG with linoleic acid in the *sn*-2 position represented 46–49% of the total seed TAG, whereas those with linolenic and oleic/vaccenic acids in the *sn*-2 positions represented 28–33% and 11–16%, respectively. In the mesocarp of Siberian, Central Asian, and Baltic berries, the major TAG species were of ACN:DB 48:1, 48:2, and 48:3 with 16:1 in the *sn*-2 position, whereas 50:1 and 50:2 with 18:1 in the *sn*-2 position dominated in the mesocarp of Caucasian berries. Caucasian berries also differed from berries from the other three sources in their lower proportion of TAG with palmitoleic acid in the *sn*-2 position. The authors suggested that sea buckthorn from the four sources could be divided into two different groups according to the different mechanisms of TAG biosynthesis in berry mesocarp. The botanical status of the berries was not defined. According to the geographical distribution of natural populations of sea buckthorn, the Siberian, Central Asian, Baltic, and Caucasian berries belong to ssp. *mongolica*, *turkestanica*, *rhamnoides*, and *caucasica*, respectively.

Vereshchagin *et al.* (22) also reported the existence of two different systems of TAG synthesis in sea buckthorn fruit mesocarp. Berezhnaya *et al.* (23) studied changes in TAG composition in the mesocarp of developing sea buckthorn fruit.

In the present study, the molecular weight distributions of TAG in the seeds and the berries of ssp. *sinensis*, *rhamnoides*, and *mongolica* and the fruit pulp/peel of ssp. *sinensis* and *rhamnoides* were analyzed by MS and MS/MS. As shown in many applications during the past years (10–14,16,18,24–27), regardless of the relatively high deviations, the MS and MS/MS procedures applied in this study, combined with the automatic calculation program, represent a fast method for the determination of regioisomers of TAG.

TAG of ACN 54 comprised 70–80% of seed TAG (including species not listed in Table 2). The rest (20–30%) were TAG of ACN 52 (C18/C18/C16). In the whole berries, the profile of TAG molecular weight species were more diversified, because they contain TAG from both the fruit flesh/peel and seeds. TAG of ACN 48 and 50 were the major species found in the fruit pulp and peel of sea buckthorn.

Clear differences were found among the subspecies in molecular weight distribution, FA combinations, and regioisomers of TAG in both seeds and berries. This is the first time that TAG of both seeds and berries of different species of sea buckthorn have been studied and compared.

In seed TAG, palmitic acid was mainly found in the ACN 52 group. Most of the palmitic acid of the ACN:DB species 52:3, 52:4, and 52:5 of seed TAG was located in *sn*-1/3 positions regardless of the FA combinations. This is in accordance with the common knowledge of the positional distribution of FA in natural TAG of vegetable origin (28).

The two most abundant TAG regioisomers in the seeds of the three subspecies were 1(3)-linolenoyl-2-linoleoyl-3(1)-linoleoyl-*sn*-glycerols and 1(3)-linolenoyl-2-oleoyl/vaccenoyl-3(1)-linoleoyl-*sn*-glycerols. In the former species, linoleic acid, and in the latter oleic/vaccinic acid, preferentially occupied the *sn*-2 position. In the combination 18:3/18:3/18:1, 1,3-dilinolenoyl-2-oleoyl/vaccenoyl-*sn*-glycerol was the preferred regioisomer . It is generally known that linoleic acid is preferentially located in the *sn*-2 position, whereas oleic and α-linolenic acids are equally distributed in *sn*-1, -2, and -3 positions in TAG of plant origin (28). The results of the present study suggest that the positional distribution of unsaturated FA depends on the FA combination as far as specific TAG molecules are concerned.

This can be further demonstrated by comparing the positional distribution of 18:1 in TAG of ACN:DB 54:6 and 54:5. In the former group, in which the major FA combination was 18:3/18:2/18:1, 18:1 was mostly located in the *sn*-2 position of glycerol. In contrast, in the ACN:DB 54:5 group, in which the major FA combinations were 18:2/18:2/18:1 and 18:3/18:1/18:1, oleic acid did not preferentially occupy the secondary position of glycerol.

It is generally accepted that both the FA composition and the positional distribution of FA in TAG molecules play a role in the nutritional properties and physiological effect of oils and fats (6,7). FA located in the primary and secondary positions of TAG have different metabolism fates after ingestion. FA in the *sn*-2 position remain as a part of monoacylglycerols directly absorbed by the intestine, whereas those in *sn*-1/3 positions are released before absorption. The *sn*-2 FA of ingested fats and oils were largely found to be in the *sn*-2 position of chylomicron TAG. Saturated FA in *sn*-1/3 positions are inferiorly absorbed compared with those in the *sn*-2 position due to the *sn*-1/3 specific actions of digestive lipase and the formation of insoluble salts in the gut (6,7). Although TAG with *sn*-2 palmitic acid are recommended fat species for infant formula, some saturated FA in the *sn*-2 position of TAG may enhance the fat absorption and unfavorably influence

postprandial lipid metabolism in adults (29). Dietary *sn*-2 long chain polyunsaturated FA may be absorbed with high efficiency compared with those in *sn*-1/3 positions (30).

Linoleic and α-linolenic acids are essential FA in the human diet. Oleic acid is a major FA of the Mediterranean diet, and is generally considered beneficial to humans. Studies carried out *in vivo* and *in vitro* have indicated that palmitoleic acid may play an important role in human health. Information on the positional distribution of these FA in TAG of sea buckthorn provides important guidelines for applications of sea buckthorn oils. Linoleic, α-linolenic, and oleic acids together accounted for around 86% of the total FA of sea buckthorn seed oil. TAG with these FA in the *sn*-2 position constituted 75–81% of the ACN:DB species analyzed, indicating the favorable position of the oil as a dietary source of beneficial FA. Seed TAG of ssp. *mongolica* contained a higher proportion of regioisomers with α-linolenic acid in the *sn*-2 position than those of ssp. *sinensis*. In the major ACN:DB species of berry TAG analyzed, ssp. *mongolica* was the richest in regioisomers with palmitoleic and linoleic acids in the *sn*-2 position and the poorest in those with *sn*-2 oleic/vaccenic acid among the three subspecies. These differences should be considered when raw materials are selected for sea buckthorn oil for specific nutritional needs.

Industries producing food and health-related products using sea buckthorn oils often face great challenges in raw material selection due to the extremely high variation in composition of sea buckthorn oils from different sources. Often, authentication is difficult. Analysis of TAG with the fast MS and MS/MS methods described in the present study could offer a solution to this problem for the future.

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NMR Characterization of Dihydrosterculic Acid and Its Methyl Ester

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ABSTRACT: Cyclopropane FA occur in nature in the phospholipids of bacterial membranes, in oils containing cyclopropene FA, and in *Litchi sinensis* oil. Dihydrosterculic acid (2-octyl cyclopropaneoctanoic acid) and its methyl ester were selected for ^{1}H and ^{13}C NMR analysis as compounds representative of cyclopropane FA. The 500 MHz ^{1}H NMR spectra acquired with $CDCl_3$ as solvent show two individual peaks at –0.30 and 0.60 ppm for the methylene protons of the cyclopropane ring. Assignments were made with the aid of 2D correlations. In accordance with previous literature, the upfield signal is assigned to the *cis* proton and the downfield signal to the *trans* proton. This signal of the *trans* proton is resolved from the peak of the two methine protons of the cyclopropane ring, which is located at 0.68 ppm. The four protons attached to the two methylene carbons α to the cyclopropane ring also show a split signal. Two of these protons, one from each methylene moiety, display a distinct shift at 1.17 ppm, and the signal of the other two protons is observed at 1.40 ppm, within the broad methylene peak. The characteristic peaks in the ^{13}C spectra are also assigned.

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FA containing a cyclopropane structure (Fig. 1; shown as methyl esters), the most common of which contain 17 and 19 carbons, occur in seed oils such as Sterculiaceae, Malvalaceae, Bombaceae, and Tiliaceae that contain cyclopropene FA, and also frequently in bacterial cell membranes (1). Among these compounds are dihydrosterculic acid (x = 7, y =7 in Fig. 1; systematic name 2-octyl cyclopropaneoctanoic acid; sterculic acid is the corresponding cyclopropene FA), lactobacillic acid (x = 9, y = 5 in Fig. 1), and dihydromalvalic acid (x = 6, y = 7). Dihydrosterculic acid has also been reported in significant amounts (41%) in *Litchi sinensis* (lychee) seed oil (2), and its isolation and absolute configuration have been reported (3).

Numerous authors (3–12) have reported not only on the synthesis of cyclopropane FA but also analytical data for these compounds, with the signals of the methylene protons in the ^{1}H NMR spectra being assigned early, including distinguishing the *cis* and *trans* protons (4,5). Selected ^{1}H NMR data contained in the literature are compiled in Table 1. The ^{1}H NMR analytical data for the salient signals do not always agree, and in some cases assignments are incomplete. Although the ^{13}C NMR signals of cyclopropenoid FA have been assigned (13,14), those of cyclopropane FA were reported but no assignments made. Therefore, in light of the significant interest in long-chain compounds containing cyclopropane moieties, commercially available dihydrosterculic acid and its methyl ester were selected for NMR analysis as representatives of the class of cyclopropane FA. Full ^{1}H and ^{13}C NMR data are reported along with assignments of key peaks.

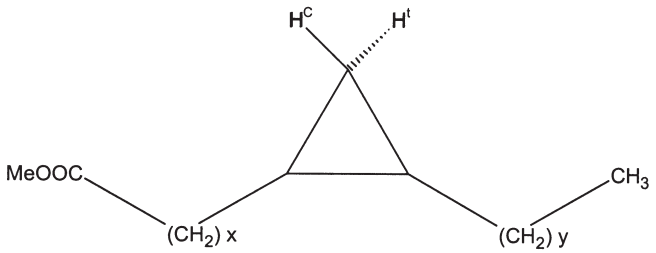

FIG 1. General structure of *cis*-cyclopropane FAME. The protons of the methylene unit in the cyclopropane are identified as *cis* and *trans* by the labels H^c and H^t, respectively.

EXPERIMENTAL PROCEDURES

Methyl dihydrosterculate and dihydrosterculic acid (synthetic material) were purchased from Matreya LLC (Pleasant Gap, PA). NMR spectra were acquired with $CDCl_3$ as solvent (approximately 40 mg sample dissolved in about 1 mL solvent) on a Bruker (Billerica, MA) Avance 500 spectrometer operating at 500 MHz (^{1}H) or 125 MHz (^{13}C). All chemical shifts are reported relative to the chloroform peak (7.29 ppm for ^{1}H NMR). Besides NMR, GC-MS analyses were carried out, using an Agilent Technologies (Wilmington, DE) 6890 GC with HP-5MS capillary column coupled to a 5973 mass selective detector, showing about 99% purity of the sample. The GC-MS results coincided well with the spectrum found by a library search (Wiley library) and literature data (3).

RESULTS AND DISCUSSION

Figure 2 depicts the ^{1}H NMR spectrum of methyl dihydrosterculate in the region of –0.5 to 2.5 ppm. The spectrum of the corresponding acid is virtually identical in this region. Expansions of the peaks at –0.30 and 0.60 ppm and the coupling constants for these signals are also shown in Figure 2.

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TABLE 1
Selected Literature Data on the ^{1}H NMR Spectra in the Range –0.5 to 2 ppm of FA or FAME Containing a Cyclopropane Ring

x; *y*; C1	Chemical Shifts[a]	Ref.
cis		
3; 17; $COOCH_3$	200 MHz; –0.38 (1H, m, *cis* H of CH_2 in cp ring); 0.54 (3H, m, *trans* H of CH_2, two C<u>H</u> in cp ring); 1.18 (32H, br, C<u>H</u>$_2$); 1.59–1.63 (4H, m, C<u>H</u>$_2$ of C4, C7), 1.67 (2H, m, C<u>H</u>$_2$ of C3)	7
4; 17; $COOCH_3$	200 MHz; –0.35 (1H, m, *cis* H of CH_2 in cp ring); 0.54 (3H, m, *trans* H of CH_2, two C<u>H</u> in cp ring); 1.24 (40H; (C<u>H</u>$_2$)$_{17}$$CH_3$, C<u>H</u>$_2$ of C4, C5); 1.61–1.67 (2H, m, C<u>H</u>$_2$ of C3)	7
4; 9; $COOCH_3$[b]	500 MHz; –0.32 (1H, H_a of CH_2, ddd, J = 5.2, 5.2, 4.6 Hz,); 0.55–0.60 (1H, m, H_b); 0.61–0.69 (2H, m, H of C6, C7); 1.08–1.49 (23H, m, positions 4–5 and 8–16, COO<u>H</u>); 1.64–1.71 (2H, m, 3 C<u>H</u>$_2$ of C3)	8[c]
7; 7; $COOCH_3$[d]	400 MHz; –0.33 (1H, m); 0.56 (1H, m); 0.64 (2H, m); 1.1–1.4 (24H, m); 1.62 (2H, m)	3
7; 7; COOH[e]	500 MHz; –0.36 (1H, q); 0.54 (1H, m); 0.63 (2H, br, s); 1.11 (2H, m); 1.30 (22H, m); 1.62 (2H, quint)	11
9; 5; COOH[f]	–0.28 (1H, q, CH, J = 3.7 Hz); 0.62 (3H, m, 3 × C<u>H</u>); 1.29–1.04 (22H, m, C_7H_{14}, C_4H_8); 1.61 (4H, m, 2 × CH_2)[g]	9
10; 2; $COOCH_3$	300 MHz; –0.34 (1H, m); 0.50–0.69 (3H, m); 1.25 (20H, br, s); 1.58 (2H, m)	10[c]
10; 2; COOH	400 MHz; –0.34 (1H, ddd, J_1 = 8.5 Hz, J_2 = 8.0 Hz, J_3 = 4.0 Hz); 0.54 (ddd, J_1 = 5.2 Hz, J_2 = 5.2 Hz, J_3 = 4.0 Hz); 0.64 (2H, m); 1.10–1.40 (20H, m); 1.62 (2H, quint, J_1 = 6.4 Hz)	12[c]
trans		
0; 14; $COOCH_3$	0.85 (5H; terminal CH_3; CH_2 in ring); 0.6 (methine at C2); 2.26 (methine at C3)	5
1; 13; $COOCH_3$	0.75 (C3 methine); 0.50 (C4 methine); 0.25 (CH_2 ring); 2.17 (2H at C2, dd)	5
2; 12–14; 0; $COOCH_3$	0.14–0.21 (CH_2 of ring); 0.34–0.40 (two methine protons)	5
7; 7; COOH	360 MHz; 0.11 (2H, t); 0.33 (2H, hept); 1.04–1.35 (24H, m); 1.61 (quint, 2H)	11
10; 2; $COOCH_3$	300 MHz; 0.35 (2H, m); 0.12 (2H, m); 1.24 (20H, br, s); 1.61 (2H, m)	10[c]
10; 2; COOH	400 MHz; 0.12 (2H, dd, J_1 = 6.5 Hz, J_2 = 6.5 Hz); 0.35 (dddd, J_1 = 17.2 Hz, J_2 = 8.9 Hz, J_3 = 6.3 Hz, J_4 = 4.5 Hz); 1.10–1.40 (20H, m); 1.60 (2H, quint, J_1 = 7.1 Hz)	12[c]
15, –[h]; $COOCH_3$	Five one-proton signals not assigned: 1.00, 0.90, 0.60, 0.40, –0.10	5

[a]All spectra were obtained using $CDCl_3$ as the solvent. Date for the terminal methyl group (around 0.88–0.90 ppm) of the FA chain is not included. br = broad, cp = cyclopropane, m = multiplet, s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, hept = heptet.
[b]Absolute configuration given as 6*S*,7*R*.
[c]This reference gives similar data for other compounds not listed in this table but with the same absolute configuration.
[d]Methyl dihydrosterculate; absolute configuration given as 9*R*,10*S*.
[e]Dihydrosterculic acid.
[f]Lactobacillic acid; absolute configuration given as 11*S*,12*R*.
[g]Spectrometer type not given.
[h]Ring in terminal position, no terminal CH_3 group.

Table 2 lists the peaks in both the ^{1}H and ^{13}C NMR spectra as well as their assignments.

In previous literature, the ^{1}H NMR spectra of cyclopropane FA or FAME have been shown to exhibit several upfield signals assignable to the cyclopropane moiety (4,5). Corresponding data are compiled in Table 1. The data show that compounds with the cyclopropane ring in *cis* configuration show different shifts than those with *trans* configuration. An earlier study using molecular models and resulting interpretation showed that *trans* cyclopropane compounds do not show the upfield peak at about –0.3 to –0.35 ppm, because in this case the methylene protons in the cyclopropane ring would be equivalent (4). The same study (4) also details earlier disagreeing assignments of the protons in the cyclopropane moiety. The downfield peak at –0.3 to –0.35 ppm can be assigned to the *cis* proton of the methylene moiety in the cyclopropane ring. The majority of data for the *cis* compounds imply that a second peak at about 0.55–0.60 ppm exists, assignable to the *trans* proton of the cyclopropane methylene unit, which agrees with earlier literature (4,5). For the *trans* compounds, two data entries given in Table 1 agree on a peak at 0.11–0.12 ppm and a peak at 0.33–0.35 ppm, although the patterns differ.

However, some data show an overlap with a peak slightly downfield (0.60–0.70 ppm) caused by two protons. Also, the data do not give the same patterns of the various peaks. Furthermore, the methylene protons in the FA chain were usually reported to be contained in the usual broad methylene peak at 1.1–1.5 ppm, with the exception of a separate peak around 1.60–1.70 ppm caused by two protons (assigned to C3 in the chain, relative to the carboxyl moiety [7]). In one case (11), however, an unassigned separate multiplet at 1.11 ppm caused by two protons was reported.

To clarify this matter, in the present work, the ^{1}H (500 MHz) and ^{13}C spectra of dihydrosterculic acid and its methyl ester (Fig. 2) as well as 2D homo- and heteronuclear correlations were acquired with $CDCl_3$ as solvent. Peaks at –0.30 ppm, 0.60 ppm, and 0.68 ppm were observed, agreeing with some literature data (3,8,12), and correlated with ^{13}C NMR shifts at 10.93 for the methylene moiety of the cyclopropane ring as well as 15.75 and 15.78–15.79 for the methine car-

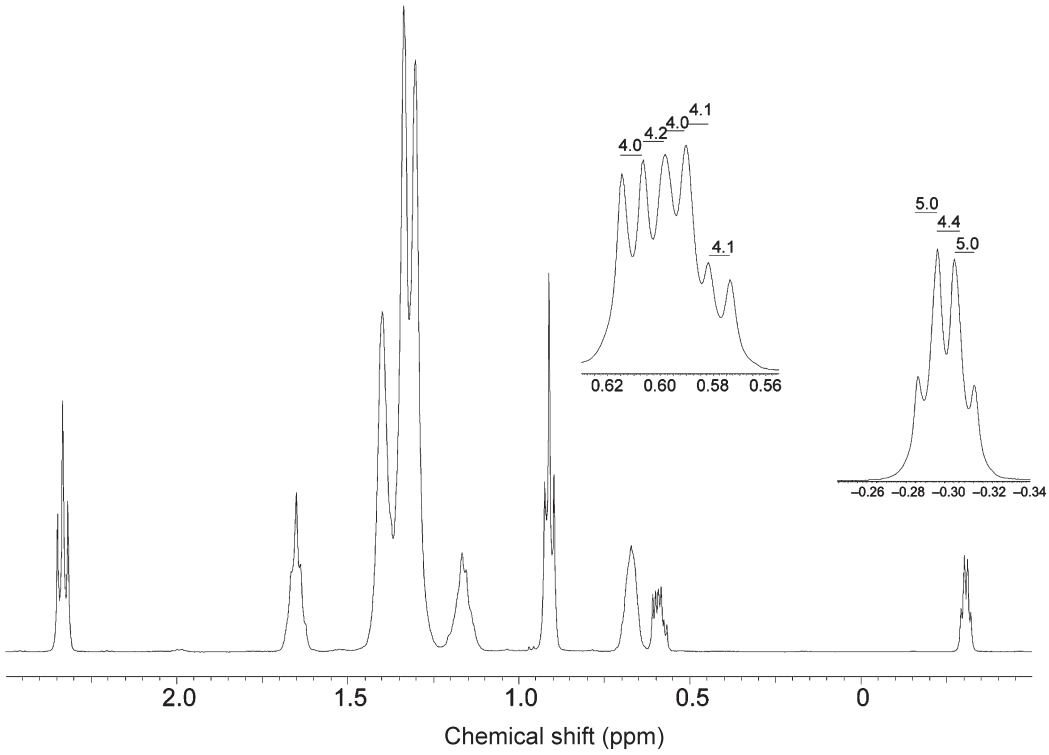

FIG 2. ^{1}H NMR spectrum of methyl dihydrosterculate (500 MHz, $CDCl_3$) in the region –0.5 to 2.5 ppm. The peaks at around 0.60 ppm and –0.30 ppm are expanded, and the coupling constants (Hz) are inscribed in these expansions.

bons. In other reports (3,7,11–12), the two peaks observed in the present work at 0.60 and 0.68 ppm apparently were not resolved. In the present work, 2D homonuclear correlation showed that the peaks at –0.30 and 0.60 ppm are assigned to the two C3 protons of the cyclopropane ring—*cis* and *trans*, respectively—and that the two methine protons resonate slightly downfield at 0.68 ppm.

The methylene peaks at 1.65 ppm and 1.17 ppm need to be investigated also. In agreement with most data in Table 1, the peak at 1.65 ppm is caused by only two protons. Two-dimensional homonuclear correlation shows clear correlation with the triplet caused by the protons attached to C2, indicating that this signal is assigned to the C3 methylene protons. On the other hand, the peak at 1.17 ppm, which was not resolved or listed separately in the previous literature, with one exception (11), correlates with the signal of the methine protons at

TABLE 2
^{1}H NMR and ^{13}C NMR Peaks with Key Assignments of Dihydrosterculic Acid ($C_{19}H_{36}O_2$) and Its Methyl Ester ($C_{20}H_{38}O_2$)

	^{13}C-NMR		
^{1}H-NMR[a]	Acid	Methyl ester	Assignment
–0.3 (1H, *cis*; q), 0.6 (1H, *trans*; ddd)	10.93	10.93	CH_2 of cyclopropane ring
0.92 (3H; t)	14.11	14.11	terminal CH_3
0.67 (2H; br)	15.75, 15.79	15.75, 15.78	CH carbons and protons (C9, C10)
1.25–1.45 (22H; br)[b]	22.71	22.70	CH_2 α to terminal CH_3 (-$\underline{CH_2}$-CH_3; ω-2)
1.65 (2H)	24.70	24.98	MeOOC-CH_2-$\underline{CH_2}$ or HOOC-CH_2-$\underline{CH_2}$
1.17 (2H; br), 1.40	28.68, 28.74	28.68, 28.73	CH_2 α to cyclopropane ring
1.25–1.45 (22H; br)[b]	29.09	29.18	CH_2 at C4 (MeOOC-CH_2-CH_2-$\underline{CH_2}$-)
	29.30, 29.37, 29.43	29.31, 29.37, 29.45	CH_2
	29.70	29.69	CH_2; 2 carbons
	30.13, 30.23	30.13, 30.22	CH_2
	31.94	31.94	-$\underline{CH_2}$-CH_2-CH_3 (ω-3)
2.33 (t, 2H)	34.06	34.13	HOOC-$\underline{CH_2}$- or MeOOC-$\underline{CH_2}$-
3.70 (s, 3H; ester only)	—	51.40	COO$\underline{CH_3}$
—		174.31	$\underline{C}$OOH or $\underline{C}$OOCH$_3$

[a]All spectra were obtained in $CDCl_3$ at 500 MHz (^{1}H NMR). For abbreviations see Table 1.
[b]This integration value contains two protons (resonating at 1.40 ppm) attached to the carbons α to the cyclopropane ring; see signal at 1.17 ppm. Also, the range 1.25–1.45 ppm appears twice to reflect the different signals in ^{13}C NMR correlating with this signal range.

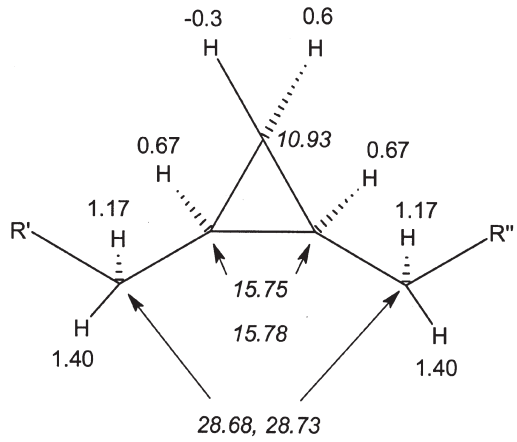

FIG 3. Assignments of chemical shifts (ppm; ^{13}C shifts are italicized) to the cyclopropane moiety in methyl dihydrosterculate. $R = -(CH_2)_6-COOMe$ and $R = -(CH_2)_6-CH_3$.

0.68 ppm and is therefore assigned to methylene protons · to the cyclopropane ring. However, the peak at 1.17 ppm is caused by two protons. In heteronuclear correlation, it correlates with ^{13}C NMR signals at 28.68 and 28.73 ppm. These two ^{13}C NMR peaks also correlate with the downfield region at 1.40 ppm, the broad methylene peak. The implication is that, likely due to shielding effects of the cyclopropane ring and similar to the differing shifts of the methylene protons in the cyclopropane ring, one proton each of the two methylene units · to the cyclopropane ring (at C8 and C11 of the FA chain) is responsible for the peak at 1.17 ppm. The downfield correlation is caused by the two other protons of these methylenes. A similar observation was made for FA with terminal cyclopentene moieties in which the signals of the methylene protons in the chain and the cyclopentene ring α to the "junction" carbon were split (15).

Table 2 contains not only information on the ^1H spectra but also on the ^{13}C spectra. The ^{13}C NMR signals not discussed above are assigned by general correlation with known data (14). The numerous methylene signals in the ^{13}C NMR spectra were not assigned to individual carbon atoms. Additionally, the assignments relating to the cyclopropane ring are depicted in Figure 3.

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Effect of Temperature on the Fatty Acid Composition and Temporal Trajectories of Fatty Acids in Fasting *Daphnia pulex* (Crustacea, Cladocera)

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ABSTRACT: Poikilothermic organisms accumulate highly unsaturated FA (HUFA) in their lipids at reduced temperatures to maintain cell membrane fluidity. In this study we investigated the effect of temperature on temporal trajectories of FA of fasting *Daphnia pulex* cultured on a HUFA-free diet. *Daphnia pulex* populations were maintained for 1 mon at 22 and 11°C and were fed the chlorophyte *Ankistrodesmus falcatus*. We observed conversion of C_{18} FA precursors to EPA (20:5n3) and arachidonic acid (ARA; 20:4n6) in *D. pulex*. We showed that long-term exposure to cold temperature causes a significant increase in EPA. HUFA such as ARA and EPA are highly conserved during starvation. Therefore, *D. pulex* has the biosynthetic capacity to adjust and to maintain the content of HUFA required to survive at low temperatures.

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Species in the genus *Daphnia* are eurythermal organisms with a wide geographical distribution (1) that exposes them to dramatic changes in temperature. *Daphnia pulex*, for example, can be cultivated at constant temperatures ranging between 2°C and 30°C (2). Many poikilothermic animals adapt to changing environmental temperatures by modifying the degree of unsaturation of their lipids (3,4). Highly unsaturated FA (HUFA) are defined as PUFA that have more than three double bonds and carbon chain lengths of C_{20} or longer (5). The high degree of unsaturation of HUFA imparts to them the property of low m.p. The accumulation of HUFA in the lipids of poikilothermic organisms at reduced temperatures can therefore be explained as an adaptation to maintain cell membrane fluidity and thus membrane function.

When *Daphnia* feed on diets containing HUFA, for example, EPA (20:5n3) or arachidonic acid (ARA; 20:4n6), their lipids become enriched with these FA (6,7). However, *Daphnia* appear unable to accumulate large amounts of docosahexaenoic acid (DHA; 22:6n3), which they readily convert to EPA (6,8). *Daphnia* can use α-linolenic acid (ALA; 18:3n3) and linoleic acid (LIN; 18:2n6) as precursors for the synthesis of EPA and ARA, respectively (6,9,10). However, *de novo* rates of FA synthesis in *Daphnia* have been shown to be less than 2% (11).

Short-term (48 h) exposure to cold temperatures (4–5°C) did not cause appreciable changes in HUFA levels of *D. pulex* (8) or *D. magna* (9). These observations indicate either that *Daphnia* exposed to cold temperatures must be highly dependent on dietary HUFA, or that enzymes involved in the HUFA synthesis need longer adaptation periods to reach full activity. We hypothesize that *D. pulex* has the biosynthetic capacity to adjust and maintain the HUFA content necessary to survive at low temperatures. Bychek *et al.* (12) recently showed that cultures of *D. magna* are able to tolerate brief (24 h) spells of fasting with very little change in lipid metabolism. Here we investigate the effect of temperature on temporal trajectories of FA of fasting *D. pulex* (hereafter referred to as *Daphnia*) grown exclusively on a HUFA-free diet.

MATERIAL AND METHODS

Culture conditions. The chlorophyte *Ankistrodesmus falcatus* (AF; strain UTCC63) was cultured in modified CHU-10 medium (13). Algae were cultured in semicontinuous chemostats at 22°C under both a General Electric Cool-White® 40-watt and a Phillips F40T12 40-watt Plant-and-Aquarium® fluorescent lamp with a combined emission of 220 μmol m^{-2} $s^{-1}$of photosynthetically active radiation. Specimens of *Daphnia pulex* (Carolina Biological Supply Company, Burlington, NC) were cultured in glass aquaria at 22°C (DA22) or 11°C (DA11) under a 12-h light–12-h dark cycle, and the *D. pulex* were fed *ad libitum* with AF that were concentrated by centrifugation before feeding. *Daphnia* were maintained under these constant conditions for 1 mon prior to their use in starvation experiments.

Starvation experiments. Tap water was preconditioned to remove chlorine and ammonia and was filtered through a 0.2-μm Sartorius membrane filter to remove most bacteria. *Daphnia* were separated from algae by pouring them onto a 350-μm mesh nylon sieve and by repeatedly rinsing them with filtered water to finish the cleaning process. Clean *Daphnia* were kept in filtered water for 24 h to provide a standard time for hind-gut clearance. *Daphnia* were counted and distributed equally among 500-mL flasks filled with 400 mL of filtered

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Abbreviations: AF, *Ankistrodesmus falcatus*; ALA, α-linolenic acid; ARA, arachidonic acid; DA11, *Daphnia pulex* cultured at 11°C; DA22, *Daphnia pulex* cultured at 22°C; HUFA, highly unsaturated FA; LIN, linoleic acid; MUFA, monounsaturated FA; TFA, total FA content.

water to obtain homogenous groups. Nongravid mid-sized animals (subadults) were selected to avoid the release of neonates during the experiment (14). *Daphnia* that were pre-adapted to 11 and 22°C for 1 mon had a similar average dry weight on Day 0 of the starvation experiment (17.7 and 18.1 μg/animal, respectively). Twenty-one flasks were stocked with *Daphnia* that were pre-adapted to 11°C (75 animals/flask). Twenty-four flasks were stocked with animals that were pre-adapted to 22°C (100 animals/flask). *Daphnia* were starved at original culture temperatures under constant dim fluorescent lighting conditions. Each day, three flasks were chosen randomly, and live *Daphnia* were collected from them. All animals were counted to determine the survival rate. When the survival rate fell below 50%, the experiments were terminated to guarantee a sufficient tissue mass for FAME analysis. DA22 could be collected only from 12 flasks because the survival rate decreased abruptly to 16.4% on Day 4. The average survival rate for DA11 was 85.8% on Day 6. *Daphnia* were frozen (at −85°C) after removing as much water as possible so as to minimize bacterial contamination, then they were freeze-dried and weighed prior to FA analysis.

FAME analyses. FAME of algae and *Daphnia* were extracted by grinding freeze-dried tissues in 2:1 (vol/vol) chloroform/methanol (15). FA were transmethylated with boron trifluoride in methanol (14% w/w). FAME concentrations were quantified on a Hewlett-Packard 6890 gas chromatograph with the following configuration: splitless injection; column = Supelco SP-2560 100 m × 0.25 mm i.d. × 0.20 μm thick film; oven = 140°C (hold for 5 min), then to 240°C at 4°C min^{-1}, hold for 12 min; carrier gas = helium, 1.2 mL/min; detector = FID at 260°C; injector = 260°C; run time = 42 min/sample.

Statistical analysis. All results are presented as mean ± SE. Independent samples collected on Day0/1, Day2/3, and Day4/5 were grouped prior to one-way ANOVA. ANOVA was followed by a Tukey test when a significant ($P < 0.05$) difference was found. Percentage data were arcsine transformed (16).

RESULTS AND DISCUSSION

Effects of growth temperature on the FA composition of Daphnia that were fed Ankistrodesmus falcatus. The FA compositions of AF, DA11, and DA22 are presented in Table 1. The lipid content of AF was 26% of its dry weight. HUFA as ARA, EPA, or DHA could not be detected in lipids extracted from AF. However, with 44.3% ALA and 15.9% LIN, total FA content (TFA) of AF contained two important precursors required for the synthesis of HUFA. AF was therefore a suitable diet to investigate the biosynthetic capacity for HUFA in *Daphnia*. In contrast to their diet, *Daphnia* grown at 11 and 22°C contained HUFA (ARA and EPA). The content of EPA was clearly influenced by temperature, because a significantly higher level of EPA was found in DA11 (12.7%) compared with DA22 (3.1%). *Daphnia* are clearly able to increase the proportion of EPA in their lipids to maintain cell membrane fluidity at low temperatures. However, for optimal growth and reproduction, additional dietary EPA might be required (17,18).

TABLE 1
Effects of Growth Temperature on FA Composition (% of total identified FAME) of *Daphnia pulex* Fed *Ankistrodesmus falcatus*

		DA22		DA11	
	A. falcatus	Mean	SE	Mean	SE
11:0	nd	nd		0.3	0.2
12:0	0.6	0.2	0.0[a]	1.0	0.2[a]
14:0	0.3	1.4	0.1[a]	1.7	0.1[a]
15:0	0.1	0.8	0.0[a]	0.5	0.0[b]
16:0	20.4	18.6	0.5[a]	19.1	0.6[a]
16:1n-7	1.0	3.3	0.1[b]	13.0	0.4[a]
17:0	0.1	0.9	0.0[a]	1.1	0.0[a]
18:0	0.5	8.1	0.5[a]	6.2	1.7[a]
18:1n-9*t*	nd	0.1	0.0[a]	0.1	0.0[a]
18:1n-9*c*	11.6	19.2	0.5[b]	23.2	0.5[a]
18:2n-6*c*	15.9	14.3	0.3[a]	6.8	1.2[b]
20:0	nd	0.3	0.1	nd	
18:3n-6	1.4	1.2	0.0[a]	0.7	0.0[b]
20:1n-9	nd	0.1	0.0	nd	
18:3n-3	44.3	22.4	0.3[a]	11.3	0.2[b]
21:0	nd	0.1	0.0	nd	
20:2	nd	1.8	0.2	nd	
22:0	2.4	0.5	0.1	nd	
20:3n-6	nd	0.1	0.0[a]	0.1	0.0[a]
22:1n-9	nd	0.3	0.0	nd	
20:3n-3	nd	0.2	0.0	nd	
20:4n-6	nd	2.7	0.3[a]	2.1	0.1[a]
24:0	0.9	0.2	0.0	nd	
20:5n-3	nd	3.1	0.2[a]	12.7	0.4[b]
24:1n-9	0.2	0.3	0.3	nd	
Σn-3	44.3	25.6	0.3[a]	24.0	0.6[a]
Σn-6	17.4	18.2	0.5[a]	9.7	1.3[b]
ΣSAFA	25.4	31.0	1.0[a]	30.0	2.2[a]
ΣMUFA	12.9	23.3	0.4[b]	36.3	0.4[a]
ΣPUFA	61.7	45.7	0.5[a]	33.7	1.8[b]

[a]Results are means ± SE. Values within a row with a different superscripted letter are significantly different ($P < 0.05$). nd = not detected. DA22, *Daphnia pulex* cultured at 22°C; DA11, *D. pulex* cultured at 11°C; SAFA, saturated FA; MUFA, monounsaturated FA.

ARA is an important precursor for signaling molecules including prostaglandins, prostacyclins, and thromboxanes (5). In contrast to EPA, DA11 and DA22 contained similar levels of ARA (2–3%). Therefore, the biosynthesis of ARA is unaffected by the difference in temperature within this range. We suggest that EPA and ARA were produced by conversion of dietary LIN and ALA precursors, especially at the lower temperature. In nature, *Daphnia* might therefore play an important role in trophic upgrading (19) of FA, thereby improving the nutritional quality of algae for subsequent use by higher trophic levels (e.g., invertebrate predators and fish). We cannot exclude the possibility that ultratrace amounts of dietary HUFA or HUFA of bacterial origin (gut bacteria) were accumulated by *Daphnia*. However, it is unlikely that EPA, at levels greater than 10% of TFA, would be derived from bacteria, particularly as care was taken to remove bacteria from the water and to clear the gut of the *Daphnia*. DHA was not detected in these *D. pulex*, confirming the results of Bychek *et al.* (12) and Farkas *et al.* (9) on *D. magna*.

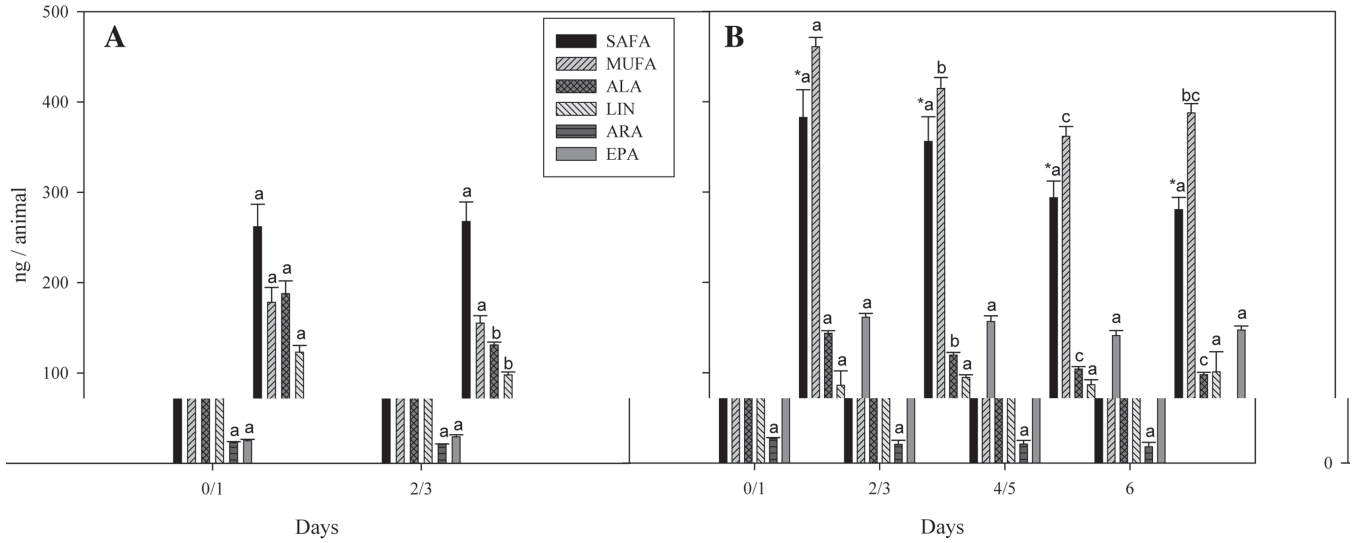

FIG. 1. Temporal trajectories of FA in fasting *Daphnia pulex* kept at (A) 22 and (B) 11°C. Results are means ± SE. Significant differences between means were determined by one-way ANOVA followed by Tukey's multiple comparison test. Values of the same FA with a different superscripted letter are significantly different ($P < 0.05$). An asterisk indicates that there was a statistically significant difference ($P = 0.031$), but the power of the performed test (0.56) was below the desired power of 0.80. ALA, α-linolenic acid; ARA, arachidonic acid; LIN, linoleic acid; MUFA, monounsaturated FA; SAFA, saturated FA.

DA11 had a significantly higher content of monounsaturated FA (MUFA), 36.3%, compared with DA22, which had 23.3%. The higher level of MUFA in DA11 might be explained by cold-induced expression of Δ9-desaturase as observed in carp (20), and can be described as a further adaptation to maintain cell membrane fluidity because MUFA have a significantly lower m.p. compared with their saturated analogs (21).

Effect of temperature on temporal trajectories of FA in fasting Daphnia. DA11 had a higher TFA on Day 0/1 (1272 ng/animal) compared with DA22 (821 ng/animal). The lower TFA in DA22 might be explained by the higher metabolic rate compared with DA11 ($Q_{10} = 2$; ref. 22). The term Q_{10} is defined as the increase in the rate of metabolic activity caused by a 10°C increase in temperature (23). If the rate doubles, Q_{10} is 2. TFA values decreased during starvation to 1061 and 779 ng/animal in DA11 and DA22, respectively. The insignificant decrease of TFA in DA22 during starvation suggests that only minor lipid reserves were available, which is confirmed by the high mortality of these animals within the first 3 d. ARA and EPA are important structural components of membrane glycerolipids, and were highly conserved during starvation in both DA11 and DA22 (Fig. 1). This agrees with Kainz *et al.* (24), who noted that EPA and ARA are highly retained in zooplankton. ALA decreased significantly in both treatments after being metabolized in order to resist starvation or after conversion to EPA. A significant decrease was also observed for LIN in DA22; however, no changes were observed in DA11.

In nature, periods of food shortage occur that can, temporarily, result in starvation conditions for zooplankton (25–27). Several effects of starvation on the biochemical composition of *Daphnia* have been described (14,28). However, we show, for the first time, trajectories of FA concentrations in starved animals under different temperature regimes. After further laboratory calibration, characteristic levels of selected FA in fasting *Daphnia* might be better defined, providing more quantitative assessments about the condition of field-derived *Daphnia* specimens.

FA can be used as trophic markers of dietary composition (29). However, our data indicate that such studies require detailed information about the effect of temperature on the extent of dietary FA conversion in different organisms.

Conclusions. We showed that long-term exposure of *D. pulex* to cold temperature causes a significant increase in EPA. HUFA, such as ARA and EPA, are highly conserved during starvation. Therefore, *D. pulex* has the biosynthetic capacity to adjust and to maintain the content of HUFA required to survive at low temperatures.

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Effects of Storage Time and Added Antioxidant on Fatty Acid Composition of Red Blood Cells at –20°C

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ABSTRACT: The stability of PUFA in venous red blood cells (RBC) of women aged 25 to 55 years (n = 12) was investigated during storage at –20°C. The RBC sample from each participant was divided into seven portions: one baseline with the antioxidant BHT, another without BHT, samples without BHT stored for 2, 4, 9, or 17 wk, and samples with BHT stored for 17 wk. No difference was found in proportions of PUFA at baseline and after storage for 2 and 4 wk without BHT, and 17 wk with BHT. After 9 wk without BHT the proportion of 22:6n-3 in RBC was lower, and after 17 wk without BHT proportions of all PUFA were lower than at baseline. High proportion of 22:6n-3 in RBC at baseline was associated with more stable concentration of total FA in RBC without BHT during 17 wk. The findings indicate that PUFA in RBC from healthy women are stable at –20°C for 4 wk without BHT and for at least 17 wk with BHT.

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Red blood cell (RBC) membrane lipids are a good indicator of dietary fat intake (1,2), as well as a biomarker of disease risk (3,4,5). RBC membrane is rich in PUFA, and a mature RBC is filled with hemoglobin containing iron, which might induce production of free radicals and lipid peroxidation of RBC membrane lipids (6).

Some centers for health service have access to only a –20°C or –30°C freezer, and human studies have shown that venous RBC stored at –20°C without addition of antioxidant had unchanged FA composition after 4 wk compared to baseline (7). Storage of RBC samples for more than 4 wk before FA analysis is often unavoidable, but lower proportion of long-chain PUFA has been found after 6 mon compared to baseline (8). The stability of PUFA in RBC may be affected by the physiological or pathological state of the individual. PUFA in RBC from healthy individuals were more stable during storage at –20°C than PUFA in RBC from patients with schizophrenia (9) and autism (10). Addition of the antioxidant BHT to RBC samples before freezing has been shown to preserve the PUFA composition for 1 yr at –50°C (7) and for 2 yr at –80°C (8) in healthy individuals.

The present study was undertaken to investigate at –20°C whether PUFA composition of RBC is stable for more than 4 wk without addition of antioxidant, and if addition of BHT preserves the PUFA content even longer.

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Abbreviations: MUFA, monounsaturated FA; RBC, red blood cells; SFA, saturated FA.

MATERIALS AND METHODS

Subjects. Nonfasting venous blood samples from healthy women (n = 12) aged 25 to 55 yr were collected in tubes containing EDTA. RBC were isolated immediately by centrifuging whole blood at 1300 × g for 10 min at 4°C, and washed three times with isotonic saline solution. The RBC sample from each participant was divided into seven portions: one baseline sample with and another without addition of the antioxidant BHT, samples without BHT stored at –20°C for 2, 4, 9, or 17 wk, and one sample with BHT stored at –20°C for 17 wk. BHT dissolved in methanol (500 mg/L) was added to RBC sample at a final concentration of 42 mg/L. No methanol was added to the RBC samples without BHT. Baseline RBC samples with or without BHT were lipid-extracted within 3 h of collection, and FA were analyzed.

Analysis of RBC total lipid fatty acids. RBC total lipids were extracted as described by Bligh and Dyer (11) except isopropanol was used instead of methanol (isopropanol/chloroform 2:1, v/v). BHT (50 mg/L) was added to the extraction medium. The FA were transmethylated for 45 min at 110°C using 14% boron trifluoride/methanol (Sigma Chemical Co., St. Louis, MO). The FAME were analyzed using GC (Agilent 6890 N, Agilent, Palo Alto, CA) equipped with a Chrompack CP-SIL 8CB column (25 m × 250 μm i.d. × 0.12 μm film thickness). The oven temperature was programmed to have an initial temperature of 150°C for 4 min, then rising 4°C/min to 230°C, then 20°C/min to 280°C, and then held isothermal for 4 min. The injector and detector temperatures were maintained at 280°C and 300°C, respectively. Hydrogen was used as the carrier gas. The FAME peaks were identified and calibrated against those of commercial standards (Sigma Chemical Co.; Nu-Chek-Prep, Elysian, MN). PC diheptadecanoyl (17:0) and heneicosanoic acid (21:0) methyl ester were used as internal and external standards, respectively. The intra- and interassay CV for analytical variation (measurement error) were, respectively, 1.13% and 1.46% for 18:2n-6, 2.09% and 1.95% for 20:4n-6, 2.38% and 2.08% for 22:6n-3, 1.29% and 1.09% for 16:0, 1.78% and 0.85% for 18:0, and 0.91% and 0.92% for 18:1n-9.

Statistical analysis. The effect of storage time and added antioxidant, and interaction between storage time and added antioxidant, were evaluated with the ANOVA for repeated measures and Tukey–Kramer *post-hoc* test in the SAS program (Mixed procedure in SAS for Windows, V8). $P < 0.05$ was considered significant. Multiple linear regression was used to evaluate the association of age or proportion of indi-

TABLE 1
Effects of Storage Time at –20°C and Addition of the Antioxidant BHT on FA Proportions (% of total FA) in RBC Total Lipids of Healthy Women[a]

			Storage time at –20°C				
FA	Baseline + BHT (n = 8)	Baseline (n = 12)	2 wk (n = 11)	4 wk (n = 12)	9 wk (n = 11)	17 wk (n = 12)	17 wk + BHT (n = 11)
Total SFA	40.47 ± 0.68	39.34 ± 0.95	40.00 ± 0.96	42.78 ± 1.64	44.38 ± 2.68[b]	50.43 ± 8.72[b]	40.42 ± 2.24
16:0	20.02 ± 0.99	19.46 ± 0.87	20.06 ± 0.80	21.37 ± 1.20	21.91 ± 1.48	26.01 ± 4.81[b]	20.09 ± 1.07
18:0	15.25 ± 0.64	14.85 ± 0.77	15.01 ± 0.86	16.29 ± 1.50[b]	16.49 ± 1.73[b]	17.02 ± 2.06[b]	15.51 ± 1.79
22:0	1.44 ± 0.20	1.34 ± 0.19	1.28 ± 0.21	1.36 ± 0.14	1.58 ± 0.15	1.99 ± 0.65[b]	1.29 ± 0.15
24:0	3.70 ± 0.46	3.43 ± 0.42	3.33 ± 0.42	3.48 ± 0.26	4.09 ± 0.47	5.09 ± 1.55[b]	3.28 ± 0.42
Total MUFA	16.91 ± 0.60	16.70 ± 0.46	16.92 ± 0.77	17.25 ± 0.83	17.74 ± 0.65[b]	19.51 ± 1.72[b]	16.50 ± 0.88
18:1n-9	12.34 ± 0.78	12.09 ± 0.55	12.38 ± 0.45	12.53 ± 0.54	12.53 ± 0.58	13.87 ± 1.20[b]	12.00 ± 0.75
18:1n-7	1.01 ± 0.08	1.06 ± 0.15	1.08 ± 0.18	1.19 ± 0.18	1.22 ± 0.14[c]	1.27 ± 0.13[c]	1.14 ± 0.25
24:1n-9	3.56 ± 0.51	3.44 ± 0.35	3.33 ± 0.30	3.35 ± 0.36	3.81 ± 0.36	4.18 ± 0.63[b]	3.25 ± 0.33
Total n-6 PUFA	26.69 ± 1.79	25.62 ± 3.07	25.25 ± 3.18	24.54 ± 2.91	23.03 ± 2.92	17.42 ± 6.14[b]	25.03 ± 3.93
18:2n-6	9.08 ± 1.03	9.12 ± 1.10	9.16 ± 1.10	9.00 ± 0.99	8.50 ± 0.94	7.25 ± 1.83[b]	8.96 ± 1.24
20:4n-6	13.63 ± 1.29	12.81 ± 1.80	12.44 ± 1.97	12.05 ± 1.83	11.30 ± 1.63	8.10 ± 3.19[b]	12.50 ± 2.33
20:3n-6	1.67 ± 0.38	1.53 ± 0.39	1.52 ± 0.42	1.47 ± 0.40	1.36 ± 0.36	0.92 ± 0.66[b]	1.49 ± 0.42
22:4n-6	2.30 ± 0.37	2.15 ± 0.63	2.13 ± 0.65	2.02 ± 0.57	1.87 ± 0.56	1.16 ± 0.81[b]	2.08 ± 0.67
Total n-3 PUFA	11.16 ± 1.61	11.54 ± 2.71	11.24 ± 2.99	10.66 ± 2.73	9.97 ± 2.65[b]	7.20 ± 4.40[b]	11.38 ± 2.06
20:5n-3	1.21 ± 0.48	1.51 ± 1.12	1.49 ± 1.16	1.40 ± 1.03	1.35 ± 1.03	0.91 ± 1.21[b]	1.48 ± 0.97
22:5n-3	2.85 ± 0.32	2.87 ± 0.51	2.76 ± 0.53	2.66 ± 0.54	2.50 ± 0.51	1.71 ± 1.05[b]	2.84 ± 0.43
22:6n-3	7.11 ± 0.98	7.16 ± 1.24	6.99 ± 1.46	6.60 ± 1.34	6.12 ± 1.23[b]	4.57 ± 2.30[b]	7.06 ± 0.88
Total FA[d]	1052 ± 192	1178 ± 139	1122 ± 126	1058 ± 150	949 ± 196[b]	766 ± 200[b]	1082 ± 144

[a]Values are reported as mean ± SD (n = 8 to 12).
[b]Significant interaction between weeks and BHT, $P < 0.05$, compared with baseline without BHT; ANOVA for repeated measures and Tukey–Kramer *post-hoc* test.
[c]Significant effect of weeks, $P < 0.05$, compared with baseline without BHT; ANOVA for repeated measures and Tukey–Kramer *post-hoc* test.
[d]mg/L red blood cells (RBC). MUFA, monounsaturated FA; SFA, saturated FA.

vidual FA in RBC lipids at baseline with the change in concentration of total FA in RBC lipids after 17 wk of storage at –20°C without added antioxidant.

RESULTS

The FA composition of RBC at baseline and stored at –20°C for 2, 4, 9, or 17 wk with or without BHT are shown in Table 1. RBC without BHT contained lower proportion of 22:6n-3 after 9 wk, and higher proportions of all saturated FA (SFA) and monounsaturated FA (MUFA), and lower proportions of all n-6 and n-3 PUFA after 17 wk than at baseline. The concentration of total FA in RBC without BHT was lower after 9 wk of storage than at baseline (Table 1).

Figure 1 shows the concentrations of six main FA in RBC at all time points with or without BHT as a percentage of the concentration at baseline without BHT. In RBC without BHT the concentrations of 20:4n-6 and 22:6n-3 were lower after 4 wk, and the concentrations of 18:2n-6 and 18:1n-9 were lower after 9 wk of storage than at baseline. After 17 wk the concentrations of 16:0 and 18:0 were also lower than at baseline. Addition of BHT preserved the FA composition and concentration of total FA (Table 1) and prevented the decrease in concentrations of individual FA in RBC during 17 wk of storage (Figure 1).

The relationship between change in concentration of total FA in RBC after 17 wk of storage without BHT and the proportion of 22:6n-3 at baseline without BHT is shown in Figure 2. The two variables were strongly associated. Multivariate model analysis showed that age and other FA proportions in RBC at baseline had no effect on the change in total FA during 17 wk without BHT.

DISCUSSION

In the present study the PUFA composition and total FA concentration were stable in washed venous RBC from healthy women during 4 wk storage at –20°C without addition of antioxidant, which supports the findings reported previously (7). However, after 9 wk the proportion of 22:6n-3 and total FA concentration was lower, and after 17 wk without addition of antioxidant all PUFA proportions were lower than at baseline. On the other hand, addition of BHT did preserve FA proportions and concentrations of venous RBC for at least 17 wk at –20°C. BHT has been shown to preserve FA composition of RBC for at least 1 yr at lower temperature (7,8).

Studies on vulnerability of n-3 and n-6 PUFA in RBC to peroxidation have been inconsistent, some showing similar (12,13,14), others increased (15,16), and yet others decreased (17) peroxidation of RBC lipids high in n-3 PUFA compared

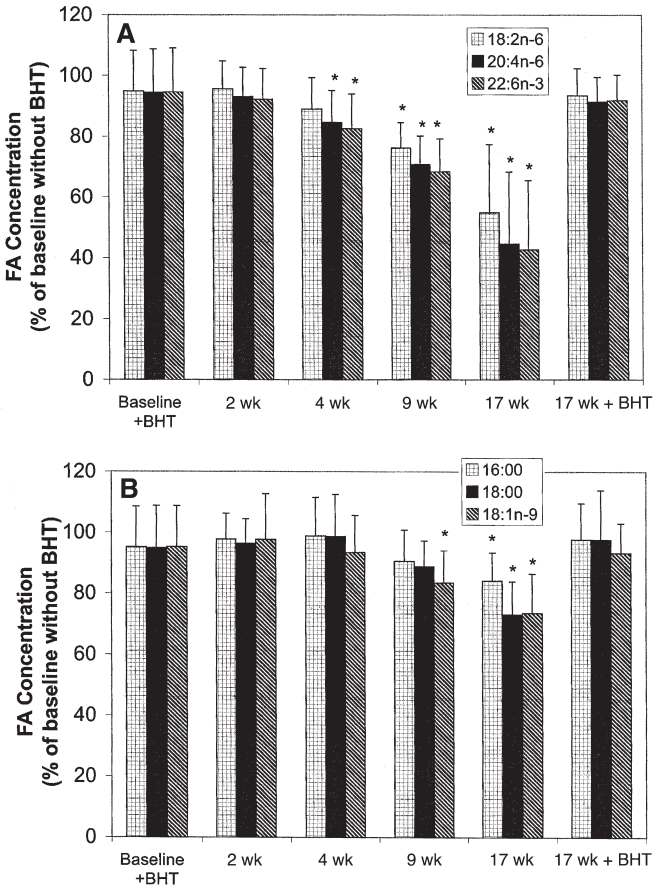

FIG. 1. Concentrations (% of baseline without BHT) of (A) 18:2n-6, 20:4n-6, and 22:6n-3 and (B) 16:0, 18:0, and 18:1n-9 in RBC of healthy women at baseline with BHT, and after 2, 4, 9, or 17 wk storage at –20°C with or without BHT. See Table 1 for number of subjects in each time point. *$P < 0.05$, compared with baseline without BHT, ANOVA for repeated measures and Tukey–Kramer *post-hoc* test.

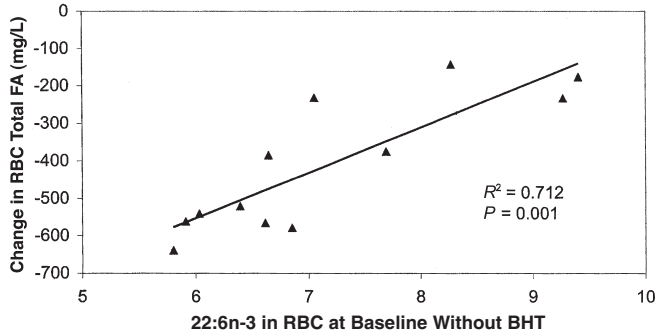

FIG. 2. Correlation between change in total FA concentration (mg/L) in red blood cells (RBC) of healthy women (n = 12) from baseline to 17 wk at –20°C without BHT and proportion (% of total FA) of 22:6n-3 in RBC at baseline without BHT.

with n-6 PUFA. In the present study, the proportion of 22:6n-3 at baseline was positively correlated to the change in concentration of total FA in RBC without BHT during 17 wk at –20°C (Fig. 2), even though n-3 PUFA deteriorated faster than n-6 PUFA (Table 1). A positive association has been found between proportion of n-3 PUFA in RBC and concentration of α-tocopherol in plasma of Icelandic women (18), and vitamin E has been shown to prevent lipid peroxidation of n-3 PUFA in animal RBC (19). Therefore we cannot exclude the possibility that antioxidants such as α-tocopherol or antioxidative enzymes naturally present in RBC may be confounding factors, and explain the correlation observed in our study. However, RBC high in n-3 PUFA have also been shown to be more resistant to hemolysis (16,20), which is one of the factors inducing lipid peroxidation of PUFA during cold storage (21). In the present study the decrease in total FA concentration during 17 wk storage without BHT was 35 ± 15% (mean ± SD), and in half the women the decrease was about 60% (over 500 mg/L, Fig. 2), where the baseline proportion of 22:6n-3 was under 7%.

In capillary RBC with low 22:6n-3 content at baseline, the decrease was 45% after 4 wk at –20°C without BHT (7). The present study indicates that the loss of RBC total FA concentration was not due only to lipid peroxidation of PUFA, as the concentration of SFA was also decreased (Fig. 1). One of the explanations for loss of total FA concentration might be that the extraction of lysophospolipids and partly degraded PL, which increase with hemolysis, was incomplete by the solvent used. Addition of BHT before storage of the RBC at –20°C prevented the degradation, probably by contributing to maintenance of the integrity of the membrane.

The findings in this study indicate that high content of 22:6n-3 in venous RBC of healthy women is associated with less deterioration of lipid FA content during storage at low temperature without addition of antioxidant. Whether RBC membranes with high content of 22:6n-3 are more resistant to deterioration in storage, because of α-tocopherol or other natural antioxidants, remains to be investigated.

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Effect of Antiretroviral Agents on Triglyceride Levels in HIV-1-Infected Pregnant Women

Sir:

Today women represent the population segment with the greatest increase in HIV infection. Despite the association between the use of antiretroviral (ARV) medications and adverse metabolic events (1–3), no study has prospectively evaluated lipid profile in pregnant women using these drugs. We carried out a controlled, prospective cohort study from September 2001 to March 2003 on 45 HIV-infected pregnant women who had not previously used ARV medications to investigate the effect of ARV on triglycerides.

The women were divided into ZDV group (zidovudine) and TT group (ZDV + lamivudine + nelfinavir) according to the criteria established by the Perinatal HIV Guidelines Working Group (4). Pregnant women with a personal history of hyperlipidemia or who were taking other medications causing secondary lipemic abnormalities were excluded. The study was approved by the Research Ethics Committee of the institution, and the subjects gave informed consent to participate. The groups were similar with respect to maternal age, body mass index, race, smoking habit, and alcohol drinking. Blood samples were obtained four times during pregnancy at equidistant time intervals after a 12–14-hour fast into a vacutainer without anticoagulant and processed in duplicate for the determination of total cholesterol and fractions and triglycerides by automated enzymatic methods using the Cobas Integra 400 apparatus of Roche (Basel, Switzerland).

Median maternal age was 24 years for the ZDV group, and 27 years for the TT group. Our findings demonstrate that TG concentration was higher in the TT group than in the ZDV group from the sixth week after the beginning of ARV treatment (Table 1).

One of the positive points of the present study was the homogeneous patient sample investigated, because several risk factors are associated with the development of dyslipidemias, with emphasis on obesity, smoking habit, alcohol drinking, and a personal and family history of diabetes mellitus and dyslipidemia. Similarly, the study was characterized by homogeneous maternal age and body mass index.

It has been demonstrated that the gestational period is associated with important phsyiological changes in lipid metabolism. The first is progressive stimulation of lipolysis and ketogenesis during pregnancy (5), and the second is a gradual increase in TG concentrations with the evolution of pregnancy, which is more constant during the third trimester of gestation, when concentrations of 1.5 to 2 times above pregestational levels occur. In the present study, TG concentration was higher in the TT group than in the ZDV group. This fact may be explained by the association between TG levels and viral burden detected in the present patient series, as also observed by Hadigan *et al.* (6). According to these investigators, this result suggests metabolic abnormalities correlated with the presence of the virus in the organism and may also represent a direct or even indirect effect of the virus, with a worsening of lipid control associated with the clinical improvement and weight gain provided by these drugs. Our study demonstrates higher elevation of TG levels in TT group. However, doubts persist about whether pregnancy might potentiate the lipid effects of ARV drugs such as the risk of pancreatitis and premature atherosclerosis. The prospective follow-up of these women on a long-term basis may permit important interventions.

TABLE 1
Triglyceride Concentration in HIV-Infected Pregnant Women at Four Periods During Pregnancy

Triglyceride	ZDV[a] (n = 20)	TT[a] (n = 25)	P^b
14–20 wk	101.1 ± 8.25	119.3 ± 11.01	0.21
21–26 wk	134.6 ± 10.90	177.1 ± 12.31	0.02
27–32 wk	146.8 ± 10.17	197.7 ± 14.92	0.01
33–38 wk	175.5 ± 13.94	226.6 ± 17.43	0.03

[a]Data are reported as mean ± SEM. ZDV, pregnant women using zidovudine; TT, pregnant women using zidovudine, lamivudine, and nelfinavir.
[b]Unpaired *t*–test.

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Differential Effects of Modulation of Docosahexaenoic Acid Content During Development in Specific Regions of Rat Brain

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ABSTRACT: Variation in brain FA composition, particularly decreased DHA (22:6n-3), affects neurodevelopment, altering visual, attentional, and cognitive functions, and is implicated in several neuropsychiatric disorders. To further understand how specific brain processes and systems are affected by variation in brain DHA content, we sought to determine whether specific brain regions were differentially affected by treatments that alter brain DHA content. Adult male Long-Evans rats were raised from conception using diet/breeding treatments to produce four groups with distinct brain phospholipid compositions. Total phospholipid FA composition was determined in whole brain and 15 brain regions by TLC/GC. Brain regions exhibited significantly different DHA contents, with the highest levels observed in the frontal cortex and the lowest in the substantia nigra/ventral tegmental area. Increased availability of DHA resulted in increased DHA content only in the olfactory bulb, parietal cortex, and substantia nigra/ventral tegmental area. In contrast, treatment that decreased whole-brain DHA levels decreased DHA content in all brain regions except the thalamus, dorsal midbrain, and the substantia nigra/ventral tegmental area. Alterations in DHA level were accompanied by changes in docosapentaenoic acid (n-6 DPA, 22:5n-6) content; however, the change in DHA and n-6 DPA was nonreciprocal in some brain regions. These findings demonstrate that the FA compositions of specific brain regions are differentially affected by variation in DHA availability during development. These differential effects may contribute to the specific neurochemical and behavioral effects observed in animals with variation in brain DHA content.

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Long-chain PUFA (LC-PUFA) are synthesized from two independent and nutritionally essential PUFA, α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6), by chain elongation and desaturation. Mammals are not able to introduce a double bond in the n-3 or n-6 position of the FA chain, but they have the enzymes to elongate the essential FA to biologically important n-3 and n-6 LC-PUFA such as EPA (20:5n-3), DHA (22:6n-3), arachidonic acid (20:4n-6), and docosapentaenoic acid (n-6 DPA, 22:5n-6). The relative availability of these LC-PUFA depends on the dietary intake of essential FA or the LC-PUFA themselves (1).

LC-PUFA are found in all cell membranes and play an important role in the structure of neuronal cells in the central nervous system, where they are in particularly high concentration. Within the lipid bilayer of the cell membrane, phospholipids containing LC-PUFA are localized preferentially around membrane proteins. Accordingly, variation in LC-PUFA composition of synaptic membranes can affect neuronal function by altering the physicochemical properties of the membrane, and thus the structure and function of receptors, transporters, and ion channels (2). LC-PUFA also serve as precursors for inter- and intracellular signals such as prostaglandins and thromboxanes, and modulate gene expression at the transcriptional level, most likely through the activation of transcription factors (3). Variation in brain FA composition, particularly decreased DHA, affects neurodevelopment, altering visual, attentional, and cognitive functions in human and animals, and is implicated in several neuropsychiatric disorders, such as schizophrenia, depression, and attention deficit hyperactivity disorder (for review see 4–9).

To further understand how specific brain processes and systems are affected by variation in brain DHA content, we sought to determine whether the phospholipid FA compositions of specific brain regions are differentially affected by treatments that alter brain DHA levels. Diet and breeding protocols were used to produce four groups of rats with distinct brain phospholipid FA compositions (10), and importantly alterations in DHA content in a range similar to those reported in clinical populations with neuropsychiatric disorders (7–9). The effects of these treatments were examined in 15 specific brain regions of adult male rats. We will show that specific regions of the rat brain have distinct FA compositions, that specific regions exhibit different changes in DHA content as a result of manipulation of DHA availability, and that the compensatory change in n-6 DPA differs between brain regions.

MATERIALS AND METHODS

Animals and diet/breeding procedures. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (11) and were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

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Abbreviations: LC-PUFA, long-chain PUFA; MUFA, monounsaturated FA; n-6 DPA, docosapentaenoic acid; SFA, saturated FA.

TABLE 1
Nutritional Composition of Diets

	Diet composition (g per kg diet)		
	ALA	ALA + DHA	Low-ALA
Basal mix formula[a]			
Casein	200	200	200
L-Cystine	3.0	3.0	3.0
Corn starch	398	398	398
Maltodextrin	132	132	132
Sucrose	100	100	100
Cellulose	50	50	50
Mineral mix[b]	35	35	35
Vitamin mix[c]	10	10	10
Choline bitartrate	2.5	2.5	2.5
TBHQ	0.014	0.014	0.014
Soybean oil	70	68.15	—
Sunflower oil	—	—	70
DHASCO[d]	—	1.85	—

[a]Harlan Teklad TD00235, composition supplied by the manufacturer. The basal mix is modified from AIN-93G (12) for use at the rate of 930 g/kg of diet prepared by adding 70 g/kg of the selected oils.
[b]Harlan Teklad TD94046 (AIN 93G-MX).
[c]Harlan Teklad TD94047 (AIN-93-VX).
[d]A triacylglycerol oil produced by algae containing 42.57% DHA by weight.

Long-Evans rats used for breeding (females >10 wk old and male proven breeders; Harlan, Indianapolis, IN) were obtained at least 5 d prior to the commencement of any treatments. Rats were housed in a temperature- and humidity-controlled animal facility with a 12-h light-dark cycle (on at 0600), given food and water *ad libitum*, and were handled regularly.

Rats were raised from conception using diet/breeding treatments designed to modulate brain DHA content. The ALA group was raised from conception on a purified diet prepared by adding 7% by weight of pure soybean oil (without partial hydrogenation) as a source of α-linolenic acid, the dietarily essential precursor of DHA, to a baseline diet (Harlan Teklad Basal Diet TD00235). The ALA diet was identical to Harlan Teklad AIN-93G and meets all current nutrient standards for rat pregnancy and growth (12). The ALA + DHA group was raised on a diet formulated with soybean oil and preformed DHA (DHASCO™, a triacylglycerol oil produced by algae containing 42.57% DHA; Martek Biosciences Corp, Columbia, MD) substituted on an equal weight basis for soybean oil such that DHA accounted for 0.7% of the total fat in the diet by weight. The low-ALA and low-ALA 2nd generation groups were the first and second generations, respectively, raised on a diet formulated with sunflower oil (7% by weight), which contains negligible α-linolenic acid compared with soybean oil. The nutritional composition of the experimental diets is presented in Table 1; FA composition of all diets, in Table 2.

Individually housed dams were placed on the respective diets at the time of initial mating. Prior to purchase, rats were fed Harlan Teklad Global 19% Protein Rodent Diet #2018S. Rats received Teklad Rodent Diet (W) #8604 for 5–7 d during acclimatization between purchase and mating. The female offspring of low-ALA dams used to generate low-ALA 2nd generation offspring (each from a different litter) were maintained on the low-ALA diet throughout their lifetime and were mated on postnatal day 70 (± 5 d). Litters were culled to 8 on postna-

TABLE 2
FA Composition of Diets

	Content in diet (g/kg)				
FA	Teklad 2018S[a]	Teklad 8604[a]	ALA[b]	ALA + DHA[b]	Low-ALA[b]
14:0[c]	0.06	0.59	0.06	0.25	0.19
16:0	7.64	5.80	6.94	7.11	4.00
18:0	1.50	1.15	2.80	2.73	2.80
20:0	0.1	0.08	0.19	0.19	0.13
22:0	0.03	0.02	0.25	0.19	0.14
24:0	0.00	0.05	0.06	0.05	0.06
16:1	0.07	0.80	0.06	0.06	0.06
18:1	12.59	8.68	14.32	14.32	12.72
20:1n-9	0.17	0.22	0.13	0.13	0.06
18:2n-6	31.25	19.67	33.73	33.09	42.89
18:3n-3	2.76	1.75	5.09	4.90	0.32
20:2n-6	0.00	0.01	0.02	0.02	0.13
20:4n-6	0.00	0.16	ND	ND	ND
20:5n-3	0.00	1.08	ND	0.05	ND
22:5n-3	0.00	0.22	ND	ND	ND
22:6n-3	0.00	0.79	ND	0.44	ND

[a]Rats were fed Harlan Teklad Global 18% Protein Rodent Diet #2018S prior to purchase and Rodent Diet (W) #8604 for 5–7 d during acclimatization. Rats were placed on the experimental diets at the time of initial mating and remained on those diets for the duration of the study. FA composition supplied by Harlan Teklad. Trace amounts of other FA reported in these formulations are not presented.
[b]Data are the total FA composition of the extracted whole diet determined quantitatively using Supelco 37 Component FAME Mix. Trace amounts of other FA detected in these formulations are not presented.
[c]Number of carbons:number of double bonds; carbons are counted from CH_3 terminal of molecule to first double bond.
ND, not detected.

tal day 1. Pups were weaned on postnatal day 21, group-housed (3 per cage), and placed on the maternal diet. On postnatal day 70, male offspring were euthanized by decapitation. Rats (n = 5 per group, each from a different litter) were used for analysis of FA composition in specific brain regions. Additional rats from the same litters (n = 3–6 per group, each from a different litter) were used for determination of whole-brain FA composition. Brains were rapidly removed, frozen on dry ice, and stored at –70°C for determination of FA composition.

FA analysis. Specific brain regions were isolated bilaterally by free-hand dissection on ice according to a modification of the method of Glowinski and Iversen (13) and weighed, a procedure taking roughly 10 min per brain. Whole-brain analyses were performed using one hemisphere of each brain. Tissues were then homogenized in methanol using a PRO250 homogenizer (4–5 mL for individual regions depending on weight, 10 mL for hemispheres). An aliquot of each homogenate was removed and extracted with chloroform-methanol (14). The methanol contained 50 mg/L of butylated hydroxytoluene as an antioxidant. The lipid extracts in organic solvents were washed with 0.15 M potassium chloride (15), and the organic solvents that remained were evaporated under nitrogen. Phospholipids were isolated from other lipid classes by TLC (silica gel G plates, 10 × 20 cm, Analtech, Inc., Newark, DE) in hexane:diethyl ether:acetic acid (80:20:1, by vol) (16). The band containing phospholipids was removed completely from the plate and transmethylated with boron trifluoride methanol (Sigma Chemical, St. Louis, MO) to yield FAME (17). Individual FAME were separated and the relative areas determined with a Varian 3400 gas chromatograph with a SP-2330 capillary column (30 m, Supelco, Belfonte, PA). The instrument was programmed for an instrument-controlled time gradient from 140°C to 208°C at the rate of 4°C/min. The injector and detector temperatures were 250°C and 300°C, respectively. Helium was used as a carrier gas at a flow rate of 21 cm/s. Individual peaks were integrated using Varian Star (v. 6) software as area percent of total FA (including plasmalogens and unidentified peaks) and identified by comparison to authentic standards (NHI-C, NHI-F, NHI-D, Supelco, Inc.; n-6 DPA, Nu-Chek-Prep, Elysian, MN) and biological mixtures (PUFA 1 and PUFA 2, Supelco, Inc.).

Data analysis. Results are presented as the mean ± SEM. Data were analyzed for statistically significant effects of the diet/breeding procedures on each FA measured by two-way ANOVA, with factors of overall brain DHA content (produced by the diet/breeding procedures) and brain region, followed by one-way ANOVA for each brain region and by Tukey's test (Systat, v. 10.2). Significance was assumed at $P < 0.05$. Regression analyses were performed using data from individual samples. Differences between slopes were determined by assessment of the confidence interval for differences between regression coefficients (18).

RESULTS

Effects of diet/breeding treatments on whole-brain phospholipid FA composition. Whole-brain total phospholipid FA composition of ALA rats was similar to that previously reported (19–22), with DHA representing 13.4% of total FA. The diet/breeding treatments used in this study produced four groups of rats with distinct phospholipid FA compositions (Table 3). DHA contents of the low-ALA and low-ALA 2nd generation groups were 77% and 66% of ALA, respectively ($P < 0.05$). In accordance with this change in DHA content, n-6

TABLE 3
Effects of Diet/Breeding Treatments on Whole Brain Total Phospholipid FA Composition[a]

	Percent of Total FA (area percent)			
Group/FA	ALA	ALA + DHA	Low-ALA	Low-ALA, 2nd Generation
16:0	17.4 ± 0.3	17.8 ± 0.3	16.7 ± 0.7	16.1 ± 0.6†
18:0	18.6 ± 0.3	19.0 ± 0.2	19.8 ± 0.4	20.0 ± 0.2
Other SFA	2.1 ± 0.2	2.3 ± 0.3	1.8 ± 0.1	1.9 ± 0.1
18:1n-7	5.2 ± 0.3	4.9 ± 0.1	6.9 ± 0.5*†	6.4 ± 0.4†
18:1n-9c	18.6 ± 0.8	19.8 ± 0.2	16.8 ± 0.5†	16.2 ± 0.6†
20:1n-9	2.2 ± 0.1	2.2 ± 0.1	2.0 ± 0.2	2.1 ± 0.2
Other MUFA	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.1
18:3n-3	0.8 ± 0.0	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.0
20:5n-3	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
22:5n-3	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
22:6n-3[b]	13.4 ± 0.2	13.4 ± 0.4	10.2 ± 0.5*†	8.8 ± 0.5*†
18:2n-6c	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.1	0.8 ± 0.1
20:2n-6	0.6 ± 0.2	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0
20:3n-6	0.5 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
20:4n-6	10.1 ± 0.4	9.2 ± 0.1	10.2 ± 0.2	11.0 ± 0.3†
22:4n-6	3.4 ± 0.1	3.0 ± 0.1	3.9 ± 0.1†	4.6 ± 0.4*†
22:5n-6[b]	0.5 ± 0.1	0.2 ± 0.0	3.9 ± 0.8*†	6.0 ± 0.3*†‡
DHA/DPA[b]	29.7 ± 4.1	70.5 ± 8.4*	2.9 ± 0.7†	1.5 ± 0.1*†

[a]Total phospholipid FA composition is expressed as the mean ± SEM (n = 3–6 per group). Data were analyzed by repeated measures ANOVA and Tukey's test. *$P < 0.05$ vs. ALA. †$P < 0.05$ vs. ALA + DHA. ‡$P < 0.05$ vs. low-ALA.
[b]Previously reported in (10).

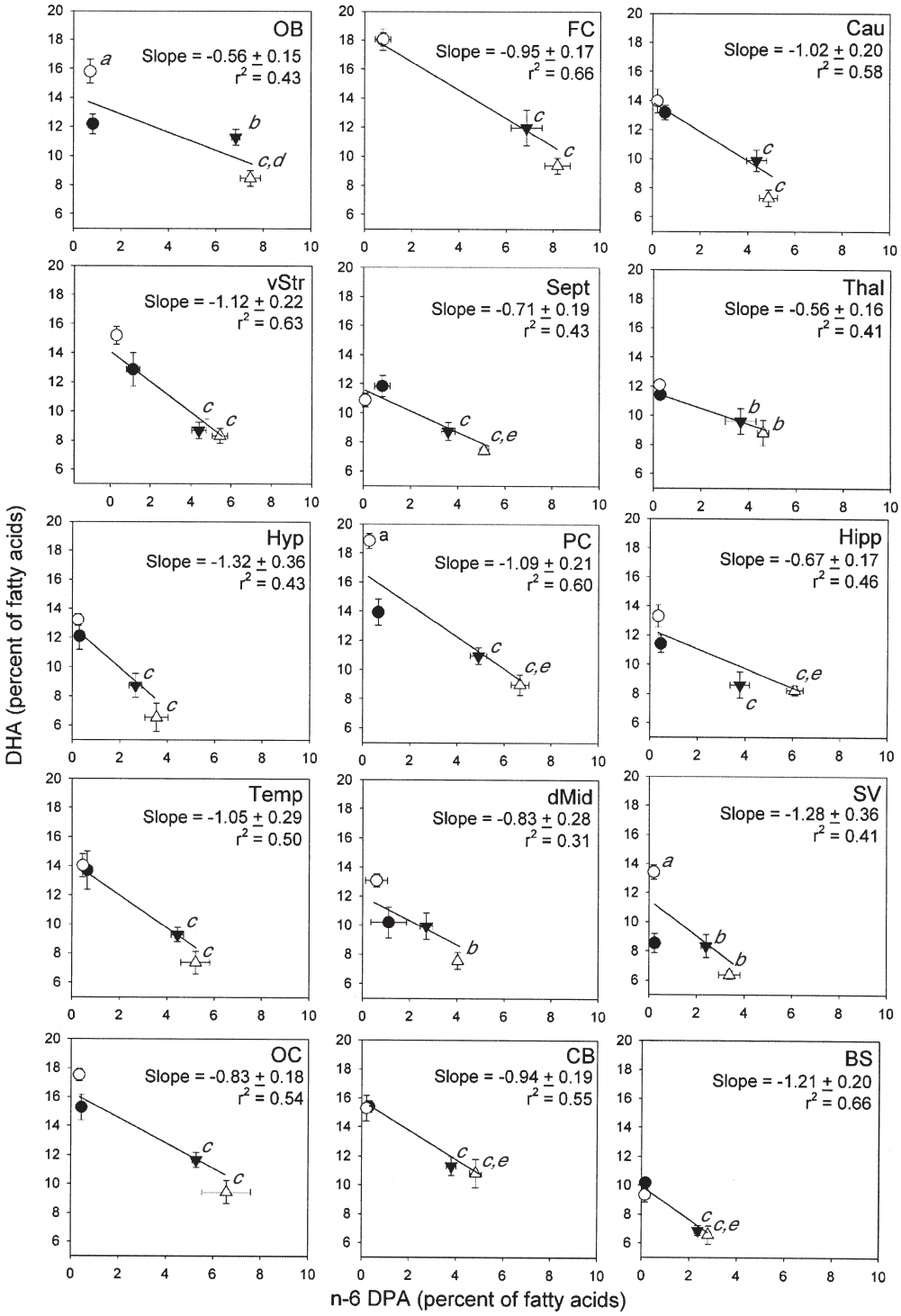

FIG. 1. Effects of diet/breeding treatments on the DHA and n-6 DPA contents of specific brain regions. Total phospholipid DHA content was determined for each of 15 brain regions of rats raised from conception using diet/breeding treatments that produced four distinct whole-brain phospholipid FA compositions. Brain regions are presented in rostro-caudal order. Data are presented as the mean ± SEM (n = 5 per group). Treatment groups: ○, ALA + DHA; ●, ALA; ▼, low-ALA; △, low-ALA 2nd generation. Significant differences by ANOVA and Tukey's test ($P < 0.05$): [a]DHA content different than ALA; [b]n-6 DPA content different than ALA; [c]DHA and n-6 DPA content different than ALA; [d]DHA content different than low-ALA; [e]n-6 DPA content different than low-ALA. For simplicity, significant differences between the ALA + DHA group and the low-ALA and low-ALA 2nd generation groups are not indicated; however, regional DHA contents in the ALA + DHA group were different than the low-ALA and low-ALA 2nd generation groups for all brain regions except low-ALA Sept, Thal, dMid, CB, and BS. Regional n-6 DPA contents for the ALA + DHA group were different than the low-ALA and low-ALA 2nd generation groups in all instances. Significant differences ($P < 0.05$) between regression slopes for some regions were detected using differences between confidence intervals of regression coefficients. OB, olfactory bulb; FC, frontal cortex; Cau, caudate-putamen; vStr, ventral striatum (nucleus accumbens and olfactory tubercle); Sept, septal area; Thal, thalamus; Hyp, hypothalamus; PC, parietal cortex; Hipp, hippocampus; Temp, temporal lobe; dMid, dorsal midbrain; SV, substantia nigra/ventral tegmental area; OC, occipital cortex; CB, cerebellum; BS, brainstem.

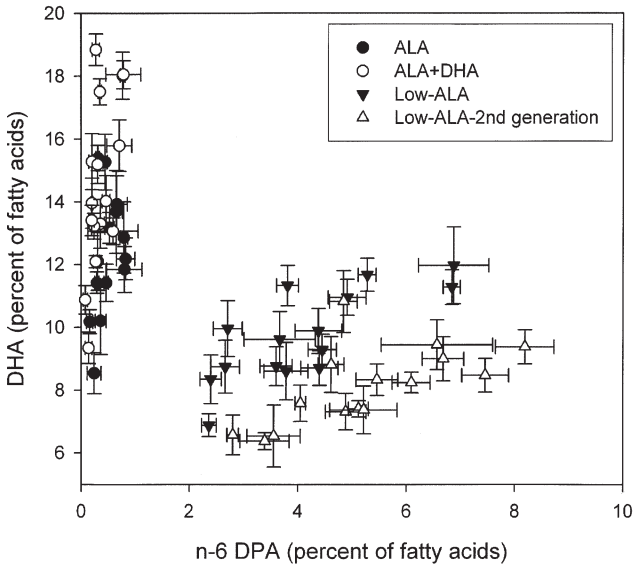

FIG. 2. Relationship between regional phospholipid DHA and n-6 DPA within treatment groups. DHA and n-6 DPA contents were determined for each of 15 brain regions. Data points represent each of the 15 individual brain regions examined expressed as the mean ± SEM (n = 5 per group). Regression analysis for ALA animals indicated a slope of 2.80 ± 0.79 (r^2 = 0.37). Slopes for ALA + DHA, low-ALA, and low-ALA 2nd generation groups were 2.27 ± 0.87 (r^2 = 0.30), 0.9 ± 0.15 (r^2 = 0.74; $P <$ 0.05 vs. ALA), and 0.60 ± 0.12 (r^2 = 0.57, $P <$ 0.05 vs. ALA and low-ALA), respectively.

DPA content was increased to 780% of ALA in the low-ALA group ($P < 0.05$) and was further increased to 1200% of ALA in the low-ALA 2nd generation group ($P < 0.05$ vs. low-ALA). In the ALA + DHA group, the ratio of DHA to n-6 DPA was significantly increased to 237% of ALA ($P < 0.05$).

Alterations in the levels of several additional FA were observed, including palmitic acid (16:0), vaccenic acid (18:1n-7), oleic acid (18:1n-9), arachidonic acid (20:4n-6), and 22:4n-6. These differences were primarily observed between the ALA + DHA and low-ALA 2nd generation groups; however, vaccenic acid in the low-ALA group and 22:4n-6 in the low-ALA 2nd generation group were different than ALA.

Regional variation in brain DHA and n-6 DPA contents in ALA rats. DHA and n-6 DPA were the primary phospholipid FA affected by the diet/breeding treatments in whole brain; thus, results for specific brain regions are presented only for these FA. In ALA rats, specific brain regions exhibited a range of DHA contents ($P < 0.05$ by ANOVA). Frontal cortex exhibited the highest DHA content (18.0%), which was roughly twofold that detected in the substantia nigra/ventral tegmental area (8.5%), the brain region with the lowest DHA content (Fig. 1). Brain n-6 DPA content also varied between brain regions ($P < 0.05$) with olfactory bulb, frontal cortex, and ventral striatum having the highest n-6 DPA contents (0.8%), which was four-fold that observed in the substantia nigra/ventral tegmental area and brain stem (0.2%). Across brain regions, DHA contents were positively correlated with n-6 DPA contents (slope = 2.80, $r^2 = 0.37$) (Fig. 2).

Effects of diet/breeding treatments on FA composition in specific brain regions. In the ALA + DHA group, the inclusion of preformed DHA in the diet resulted in significantly increased DHA content in the olfactory bulb (130% of ALA), parietal cortex (135% of ALA), and substantia nigra/ventral tegmental area (157% of ALA) (Fig. 1). DHA content of all other brain regions was not different than ALA. No significant changes in regional n-6 DPA content were detected.

In the low-ALA group, the significant (23%) decrease in whole-brain DHA content resulted in significant decreases to between 66% and 79% of ALA in 11 of the 15 brain regions examined. Frontal cortex was the most affected (66% of ALA), whereas decreases in the olfactory bulb, thalamus, dorsal midbrain, and substantia nigra/ventral tegmental area were not of sufficient magnitude to reach statistical significance. Significant increases in n-6 DPA content were observed in all brain regions where decreases in DHA were observed.

In the low-ALA 2nd generation group, the multigenerational treatment with the diet lacking α-linolenic acid resulted in a trend toward greater regional decreases in DHA content than observed in the low-ALA group; however, this further decrease reached statistical significance only in the olfactory bulb ($P < 0.05$ vs. low-ALA). n-6 DPA content was significantly increased in all brain regions compared with ALA ($P < 0.05$) and was significantly greater in low-ALA 2nd generation rats compared with low-ALA DHA rats in the septal area, parietal cortex, hippocampus, cerebellum, and brain stem ($P < 0.05$).

The relationship between regional DHA and n-6 DPA contents for each brain region across treatments was examined by regression analysis (Fig. 1). Hypothalamus (slope = –1.31) and substantia nigra/ventral tegmental area (slope = –1.28) exhibited the steepest slopes, indicating the greatest change in DHA content relative to n-6 DPA, and were significantly different ($P < 0.05$) from the olfactory bulb and thalamus (slope = –0.56), which had greater change in n-6 DPA content relative to the change in DHA content across the range of treatments. Significant differences between regression slopes ($P < 0.05$) were detected between the confidence intervals for slopes for some brain regions.

Regression analysis of the relationship between regional DHA and n-6 DPA contents within each treatment group indicates that DHA and n-6 DPA content exhibited a positive correlation (Fig. 2). The regression slopes for DHA vs. n-6 DPA in the ALA and ALA + DHA groups were not significantly different, consistent with the similar whole-brain DHA content in these groups. In agreement with the decrease in DHA content observed in whole brain and the compensatory incorporation of n-6 DPA under conditions with lower availability of DHA, the slopes for the low-ALA and low-ALA 2nd generation groups were significantly lower than the ALA slope ($P < 0.05$). Likewise, slopes for the low-ALA and low-ALA 2nd generation groups were different from each other ($P < 0.05$). However, the positive correlation within each treatment group was retained.

DISCUSSION

Adequate availability of DHA is important for optimal brain development and appears to affect visual, cognitive, and attentional processes (4–6). In addition, a growing body of epidemiological and clinical evidence suggests that low levels of n-3 LC-PUFA, particularly DHA, may contribute to the development or severity of neuropsychiatric disorders such as schizophrenia (7,8) and attention deficit hyperactivity disorder (9), with early development as a period of particular vulnerability. Accordingly, DHA was included in infant formula in 2002 and several n-3 PUFA supplements are marketed to pregnant and nursing women. Brain FA composition, including DHA content, can vary depending on the availability of specific FA (23–25); however, relatively little is known about the effects of such variation on the FA composition of specific brain regions. This study evaluated the phospholipid FA composition of specific brain regions in four groups of rats with distinct whole-brain phospholipid FA compositions.

The DHA content of rat brain observed in this study is similar to that reported in numerous previous studies (e.g., 19,20,22,26). In agreement with previous studies in rodents (27,28), the FA composition of specific regions exhibited significant variation in DHA content, with higher levels of DHA in regions such as the frontal cortex and lowest levels in regions such as the substantia nigra/ventral tegmental area. However, the regional distribution of DHA in the rodent differs from that reported in the 4-wk-old baboon (29), where the range of DHA levels in brain regions was smaller and the highest DHA levels were observed in basal ganglia, discrepancies that likely reflect both species and maturational differences.

The decrease in whole-brain DHA content in the low-ALA group in this study is similar to that observed in previous studies with rats raised from conception on diets formulated with sunflower oil (26). Decreases in brain DHA content of greater magnitude have been produced in first-generation models using diets formulated with oils such as peanut and safflower, which contain even less α-linolenic acid than sunflower oil (30). Similarly, the DHA content of the low-ALA 2nd generation group in this study, produced by breeding two generations of rats on a diet formulated with sunflower oil, resulted in a smaller decrease in DHA level than that observed in other studies using multiple-generation models (20,22,31). Again, this discrepancy is probably caused by differences in the formulation of the diets and perhaps strain differences. The decreases in DHA content in this study are similar to the 15–50% decreases relative to normal controls observed in tissues from patients with neuropsychiatric disorders such as ADHD (32,33) and schizophrenia (34–37) and are thus likely to model these clinical conditions.

In concordance with previous studies that examined the effects of decreased brain DHA in specific regions of the rodent brain (20,27,28), the present data clearly demonstrate that brain regions respond differentially to treatments that alter DHA accretion over the course of development. Under conditions of increased availability of DHA from the inclusion of preformed DHA in the diet, only three brain regions (olfactory bulb, parietal cortex, and substantia nigra/ventral tegmental area) exhibited significant increases in DHA content, a change that was not reflected in a detectable alteration in whole-brain DHA level. Under sufficiently strenuous conditions of reduced DHA availability, all brain regions except thalamus exhibited depletion of DHA content, with frontal and temporal cortices, caudate-putamen, and hypothalamus exhibiting the greatest depletion under the low-ALA 2nd generation treatment condition. Over the range of treatments examined in this study, substantia nigra/ventral tegmental area and parietal cortex exhibited the greatest range of DHA contents, whereas brain stem, thalamus, cerebellum, and septal area exhibited the least. Previous studies using multigeneration treatments with α-linolenic acid–deficient diets suggest that the variation in brain phospholipid DHA content most likely represents changes in the composition of phosphatidylethanolamine and phosphatidylserine, the phospholipid species that contain the highest concentrations of DHA (38). With regard to specific cell types, oligodendrocytes are most affected followed by neurons, whereas astrocytes are least affected (39). It is likely that these specific phospholipid subtypes and cell types are similarly affected in this study, particularly in the low-ALA 2nd generation group; however, the actual effects of the FA compositions of these phospholipid subclasses and cell types over a range of brain DHA contents and in specific brain regions have yet to be determined. Likewise, it must be noted that this study assessed the effects of variation in the availability of DHA during development in young adult rats. Differences between the regional distribution of DHA between neonatal baboons (29) and adult rodents (20,27,28) suggest that DHA accretion in specific brain regions may occur at different rates during different phases of development. Accordingly, the present observations may not be valid at time points in development other than adulthood.

Incorporation of n-6 DPA into phospholipids under conditions with decreased availability of DHA is well established (23). As expected, variation in the availability of DHA during development resulted in inversely related changes in regional DHA and n-6 DPA contents in all brain regions. However, consistent with the nonreciprocal replacement of DHA by n-6 DPA reported in rat cortex (40) and retina (41), the significant differences among regional DHA/n-6 DPA regression slopes across treatments suggests that the quantitative relationship between the incorporation/loss of DHA and the loss/incorporation of n-6 DPA differed among regions. Reciprocal substitution of n-6 DPA for DHA appears to occur in brain regions where the DHA/n-6 DPA regression slope across treatments was close to –1, such as the caudate nucleus, parietal cortex, and temporal lobe. However, the compensatory changes in these FA were not reciprocal in other regions, such as the olfactory bulb, parietal cortex, and substantia nigra/ventral tegmental area, where the increases in DHA produced by the diet containing preformed DHA were not accompanied by a commensurate decrease in n-6 DPA. On the other hand, in brain regions such as the thalamus, septal area, and hippocampus (where the regression slope was greater than –1), the low-ALA and low-ALA 2nd genera-

tion treatments produced greater incorporation of n-6 DPA than decreases in DHA. Finally, whereas the relationship between DHA and n-6 DPA across treatments appears linear for some brain regions (e.g., dorsal midbrain), this may not be the case in others (e.g., olfactory bulb).

Differences in the change in phospholipid FA composition of specific brain regions may contribute to the neurobiological effects associated with decreased availability of DHA during development. For example, function of systems associated with the midbrain, which has relatively low levels of DHA in rats raised on the ALA diet, are altered by treatments that decrease accretion of DHA during development. Substantial decreases in brain DHA content (–75%) alter function of the mesolimbic and mesocortical dopamine systems (42), and changes in motor activity, a parameter relevant to dopaminergic function, have been observed in animals with various levels of DHA depletion (e.g., 6,26,43,44) or increased DHA (45). Interestingly, a moderate decrease in brain DHA content resulted in increased locomotor activity, whereas a greater decrease in DHA resulted in decreased activity (10,26,46), perhaps caused by differing degrees of DHA depletion in different brain regions. Altered evoked visual and auditory cortical potentials are also reported in rats with a moderate decrease (–20%) in brain DHA content (47). Similarly, performance in an olfactory discrimination task was decreased in rats with a 79% decrease in brain DHA content (22,48), consistent with the depletion of DHA and substantial incorporation of n-6 DPA observed in the olfactory bulb in the low-ALA 2nd generation group. In view of the relative lack of effect of the low-ALA treatment on DHA content in olfactory bulb, it is interesting to hypothesize that olfactory function might be less affected by treatments that produce only a modest decrease in whole-brain DHA content. The specific relationship between regional FA composition and functional effects, however, must be determined by systematic study over a range of brain DHA contents.

In summary, the present findings demonstrate that the phospholipid DHA contents of specific brain regions are differentially affected by diet/breeding treatments that alter brain DHA accretion over the course of development. Compensatory changes in n-6 DPA content are also regionally specific and appear to be nonreciprocal with DHA in some brain regions. These differential effects on regional LC-PUFA composition may underlie the neurochemical and behavioral effects resulting from variation in DHA availability and suggest that different neurochemical alterations may occur with changes in brain DHA content of various magnitudes.

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Dietary Intakes and Food Sources of Fatty Acids for Belgian Women, Focused on n-6 and n-3 Polyunsaturated Fatty Acids

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ABSTRACT: The intake of fat, saturated and monounsaturated FA (SFA and MUFA), and omega-6 and omega-3 PUFA has been estimated in 641 Belgian women (age 18–39 y). Their food intake was recorded using a 2-d food diary. The PUFA included were linoleic (LA), alpha-linolenic (LNA), arachidonic (AA), eicosapentaenoic (EPA), docosapentaenoic (DPA) and docosahexaenoic (DHA) acids. The mean total fat intake corresponded to 34.3% of total energy intake (E). The mean intake of the FA groups corresponded to 13.7%, 13.1%, and 6.0% of E, for SFA, MUFA, and PUFA, respectively. The mean intake of LA was 5.3% of E and of LNA was 0.6% of E, with a mean LA/LNA ratio of 8.7. The mean intake of AA was 0.03% of E. The mean intake of EPA, DPA, and DHA was 0.04%, 0.01%, and 0.06% of E, respectively. According to the Belgian recommendations, the total fat and SFA intake was too high for about three-quarters of the population. The mean LA and overall n-6 PUFA intake corresponded with the recommendation, with part of the population exceeding the upper level. Conversely, the population showed a large deficit for LNA and n-3 PUFA. The major food source for LA and LNA was fats and oils, followed by cereal products. The main sources of long-chain PUFA were fish and seafood, and meat, poultry, and eggs. From a public health perspective, it seems desirable to tackle the problem of low n-3 PUFA intake.

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Advances in the knowledge concerning physiological functions of dietary PUFA, in particular omega-3 PUFA, have led to an increased interest in the food sources and the level of dietary intake of these nutrients. Alpha-linolenic acid (LNA, C18:3n-3) is a plant-derived omega-3 FA. Together with linoleic acid (LA, C18:2n-6), LNA is one of the two essential FA in the human diet; LA and LNA cannot be synthesised by the human metabolism. LNA can be desaturated and elongated in the human body to its longer-chain relatives, long-chain n-3 PUFA (LC n-3 PUFA), but the efficiency of this conversion is reduced by high intake levels of LA, which competes more effectively than LNA for desaturation and elongation enzymes because LA is abundantly present in Western diets (1). There is evidence to suggest that the conversion rate of dietary LNA to LC n-3 PUFA is insufficient to achieve adequate levels, even when the LNA intake is increased (2,3). Nevertheless, a British study suggested that women may possess a greater capacity for LNA conversion than men (4,5). LC n-3 PUFA are not synthesised by plants, but they are present in animals and in the marine food chain, EPA (C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3), and DHA (C22:6n-3) being the most abundant in the human diet. It is via planktivorous fishes that LC n-3 PUFA enter the marine food chain and accumulate in seafood (2). Therefore, seafood products are excellent food sources of LC n-3 PUFA.

For more than 30 years, a lot of fundamental clinical and epidemiological research work has been done with regard to the relationship between n-3 FA and health, showing the potential role of n-3 PUFA from the diet in the prevention of several diseases, in particular cardiovascular diseases (1,6,7). Moreover, the importance of an adequate intake of DHA, particularly during pregnancy, is stated, given the role of DHA in the development of visual functions and the nervous system. Because LC n-3 PUFA cannot be formed by human cells to a significant extent, most of the n-3 FA accumulated in the fetal tissue must originate from the maternal diet (8,9). The knowledge about the beneficial effects of these FA has led to the formulation of dietary reference intakes for the absolute amount of PUFA, but also for the balanced intake of n-6 and n-3. In 2003, the Belgian Health Council formulated recommendations for FA intake for the Belgian population, expressed in percentages of total energy intake (Table 1) (10). However, there is a lack of information on the current dietary intakes and sources for the Belgian population. One of the reasons for this has been the lack of a sufficiently complete food composition database (FCDB) for FA (11). Up to now, dietary intake assessments of individual n-6 and n-3 PUFA on a population level can be found for Japan (12,13), France (14), Norway (15), Australia (11,16), and Germany (17).

The aim of this study is to determine the total intake of fat, FA groups, and in particular n-6 and n-3 PUFA in a population sample of Belgian women of reproductive age, and to compare these intakes with the current Belgian recommendations (10). In addition, the main food sources of these FA have been identified.

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Abbreviations: AA, arachidonic acid; DPA, docosapentaenoic acid; E, total energy intake; FCDB, food composition database; long-chain PUFA (LC PUFA); linoleic acid (LA); alpha-linolenic acid (LNA); MUFA, monounsaturated FA; SFA, saturated FA.

TABLE 1
Recommended Daily Intakes of Fat and FA, Formulated by the Belgian Health Council (10)[a]

	Recommendations (%E)
Fat	< 30
SFA	< 10
MUFA	> 10
PUFA	5.3–10.0
18:2n-6	> 2.0
20:4n-6	—
Σn-6 PUFA	4.0–8.0
18:3n-3	> 1.0
Σ(20:5n-3, 22:6n-3)	> 0.3
Σn-3 PUFA	1.3–2.0

[a]Σn-6 PUFA = 18:2n-6 + 20:4n-6; Σn-3 PUFA = 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3; %E, percent of total energy intake.

MATERIALS AND METHODS

Population sample. The data reported in this paper are from a large epidemiological survey that included women aged 18 to 39 y. A total of 4,000 women were randomly selected from the population register of Ghent, a medium-sized city (a population of 229,000 inhabitants) in Flanders, the Dutch-speaking, northern part of Belgium. They were invited to participate in the study by postal mail and were asked to reply by use of an enclosed postcard. Not less than 2,634 subjects declined the invitation to participate, and 424 invitation letters were declared undeliverable by the postal service. Women who were pregnant, who had moved to another city, who did not speak Dutch, who were unable to come to the research center, or who were unable to volunteer within the planned period of the fieldwork were excluded from the study. In total, dietary data were collected for 641 women. The primary objective of the survey was to evaluate food and nutrient (in particular iron) intake in a group of Belgian women of reproductive age. Dietary assessment was done on the basis of a 2-d food record, using diaries with a semistructured, open-entry format and consisting of six eating occasions: breakfast, lunch, dinner, and morning, afternoon, and evening snacks. The women were asked to give a detailed description of the foods eaten, if possible to give a brand name and to estimate the amounts using natural measures (e.g., pieces, slices) or household measures (e.g., coffee spoon, cup). Experienced dietitians used a standardized protocol, including a manual on household weights and measures, to convert the estimated amounts into weights. In addition, a general sociodemographic questionnaire was administered. Data were gathered during the year 2002 (January 29 to December 22), thereby covering the different seasons. The study was approved by the Ethical Committee of the Ghent University Hospital. Only the data from the 2-d food record are used in this study for assessing the intake of total fat and important dietary FA.

FCDB. A specific FCDB was developed on the basis of existing food composition data. Of all the different food items in the study (n = 1,063), 75.1% contained fat (n = 799). Total fat contents for these foods were available from the Belgian FCDB (18) and the Dutch FCDB (19). For the establishment of a detailed FA database, including SFA, MUFA, PUFA, LA, LNA, AA, EPA, DPA, and DHA, the following eight different existing food composition tables were used: an extended version of the Dutch FCDB (20) (for 457 food items); a specific French FCDB (14) (for 115 food items); the British McCance & Widdowson's FCDB (21) (for 59 food items); the USDA National Nutrient Database (22) (for 51 food items); the Finnish FCDB (23) (for 12 food items); the Canadian Nutrient File (24) (for 9 food items); the Danish FCDB (25) (for 6 food items); and the German Food Composition and Nutrition Table (26) (for 1 food item). Furthermore, food composition information from industries was used for 43 food items, mostly specific varieties of margarine, cheese, and dressings. Finally, for 46 composite food items, the FA composition was calculated using both local recipes describing the different ingredients and their proportions, and the FA composition of the ingredients as found in one of the FCDB. Detailed FA profiles for the 799 food items were then compiled using the listed databases by applying the proportional share of each FA (group) in the total fat content as found in the database of origin, to the total fat content of the food as listed in the local FCDB (18,19).

Statistics. The population intake is calculated and expressed as means, standard deviations, and different percentiles (P5, P50, P95). All statistics are based on the mean of 2 d for each individual. The intakes are expressed in absolute amounts (g or mg per d) as well as in percentage of total energy intake. The percentages of the n-6 and n-3 PUFA provided by the different food items were calculated. These percentages were calculated as population proportions, as defined by Krebs-Smith *et al.* (27). The population proportion is calculated by summing the amount of a certain FA from a certain food item for all individuals and then dividing that by the sum of that FA from all food items for all individuals. The food items were grouped in eight different food groups, each consisting of several subgroups, as presented by Astorg *et al.* (14).

RESULTS

The intakes of total fat, SFA, MUFA, and PUFA of Belgian women (n = 461) are given in Table 2 in grams per day (g/d) and in percentage of total energy intake (%E). This table also shows the energy intake (kJ/d). The intakes of individual PUFA expressed in mg/day and in %E can be found in Table 3. The mean E of the study population was 8,338 kJ. On average, 34.3%E was provided by fat intake. The mean intake of SFA and MUFA was very similar, respectively 13.7% and 13.1%E, whereas the mean intake of PUFA was less than half of it, being 6.0%E (Table 2). Flemish women consumed on average 11.92 and 1.44 g (5.28 and 0.64%E) of LA and LNA, respectively, per day. As a consequence, the LA/LNA ratio was fairly high: 8.7 on average, with 50% of the population having a ratio over 10.1 (Table 4). The mean daily intake for AA amounted to 55.9 mg (0.03%E). Mean intakes for EPA, DPA and DHA were 77.8 mg/d (0.04%E), 25.3 mg/d (0.01%E) and 131.2 mg/d (0.06%E), respectively (Table 3). The average total n-6 PUFA intake (sum of LA and AA, presented as ∑n-6PUFA) was 11.97 g/d (5.33%E). Mean total n-3 PUFA intake (sum of LNA, EPA, DPA, and DHA, presented as ∑n-3 PUFA) was 1.67 g/d (0.75%E). As a result, the mean ratio of ∑n-6 PUFA to ∑n-3

TABLE 2
Intake of Energy, Total Fat, and the Three Different FA Groups

	Energy Intake	Total Fat	SFA	MUFA	PUFA	Total Fat	SFA	MUFA	PUFA
	kJ/d	g/d				%E			
Mean	8,338	77.6	30.9	29.6	13.4	34.3	13.7	13.1	6.0
SD	2,076	27.9	12.1	11.7	6.4	6.9	3.4	3.3	2.3
5th percentile	5,115	36.2	13.5	13.3	4.7	22.6	7.9	7.9	2.7
Median	8,243	75.3	29.2	28.4	12.6	34.4	13.7	12.9	5.7
95th percentile	11,964	125.6	52.3	50.4	25.0	45.4	19.2	18.8	10.0

TABLE 3
Intakes of Individual PUFA

	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:5n-3	22:6n-3	Σn-6 PUFA	Σ(20:5n-3,22:6n-3)	Σn-3 PUFA
	mg/d								
Mean	1,1918.8	1,436.9	55.9	77.8	25.3	131.2	1,1974.8	208.9	1,671.2
SD	5,859.5	902.6	47.4	156.1	45.7	246.9	5,869.1	396.6	1,005.4
5th percentile	3,915.0	432.2	3.6	0.0	0.0	0.0	3,938.5	0.0	532.1
Median	1,1079.3	1,258.5	43.8	14.0	11.7	42.5	11,113.8	54.1	1,445.8
95th percentile	2,2778.4	3,030.5	147.9	427.7	100.2	647.1	22,798.5	1,115.4	3,558.1
	%E								
Mean	5.28	0.64	0.03	0.04	0.01	0.06	5.33	0.10	0.75
SD	2.08	0.34	0.02	0.07	0.02	0.11	2.12	0.18	0.40
5th percentile	2.39	0.24	0.00	0.00	0.00	0.00	2.40	0.00	0.29
Median	5.04	0.57	0.02	0.01	0.01	0.02	5.07	0.02	0.66
95th percentile	9.05	1.32	0.07	0.18	0.05	0.28	9.00	0.44	1.53

For abbreviations, see Table 1.

PUFA was 7.8. The 5th and 95th percentiles of this ratio show a quite high variation (Table 4). The contribution of the LC n-6 derivates to the ∑n-6 PUFA was on average very low, only 0.5%. For the ∑n-3 PUFA, the LC fraction accounted for 14.0%. Furthermore, the LC PUFA intake is skewed to the right, as shown by the median intakes being much lower than the mean intakes (Table 3). A large proportion of the study population had no intake of the LC PUFA on both recorded days, whereas others had rather high intakes.

The percentages of fat, SFA, MUFA, total PUFA, and individual n-6 and n-3 PUFA provided by different food types are presented in Tables 5 and 6. The major food sources contributing to total fat intake were dairy products, sweet products, fats and oils, and meat, poultry, and eggs. Dairy products and sweet products were the main contributors to SFA intake, whereas fats and oils were the primary source of MUFA and PFUA (Table 5). Fats and oils were the main sources for LA (31%) and especially of LNA (45%), followed by cereal products. In the group of fats and oils, margarines and fatty sauces (dressings, etc.) were the major subgroups. Breads was the most important subgroup in the groups of cereal products. The intake of AA was mainly contributed to the diet by meat, poultry, and eggs, which contributed more than half of the intake. The subgroup eggs was responsible for more than 25% of the overall AA intake. The second important contributor was fish and seafood, accounting for 17.8%, with molluscs and crustacean as the most important subgroup, followed by fatty fish. For the three different LC n-3 PUFA taken into consideration in this study, a nearly equivalent pattern is found concerning the contribution of the different food groups to the mean intake. Fish and seafood contributed 87.3%, 66.0%, and 80.0%, respectively, of EPA, DPA, and DHA. The most important subgroup was fatty fish, which accounted for almost half

TABLE 4
Ratio of the Intake of LA versus LNA and of the Intake of n-6 PUFA versus n-3 PUFA

	18:2n-6/18:3n-3	Σn-6 PUFA/Σn-3 PUFA
Mean	8.7	7.8
SD	5.4	4.2
5th percentile	5.3	4.2
Median	10.1	8.8
95th percentile	19.0	16.4

For abbreviations, see Table 1.

TABLE 5
Food Sources for Total Fat and the Three FA Groups (% of Contribution)

Food Group	Fat	SFA	MUFA	PUFA
Total cereal products	8.9	8.7	8.2	12.3
Total dairy products	19.7	31.0	14.9	3.0
Total fats and oils	18.5	9.9	22.1	31.7
Total fish and seafood	1.7	0.9	1.7	2.8
Total fruits and vegetables	9.3	5.8	10.9	13.3
Total meat, poultry, and eggs	15.4	14.3	17.1	12.2
Total miscellaneous	7.5	4.5	8.3	12.8
Total sweet products	19.1	24.9	16.7	11.9

TABLE 6
Contribution of Food Sources to n-6 and n-3 PUFA Intakes (% of the Total Intake of Each FA Brought by Each Food Group: Means of the Whole Population Sample)

Food Groups	LA	LNA	AA	EPA	DPA	DHA	Σn-6[a]	Σn-3[b]
Breads	12.9	9.9	0.0	0.0	0.0	0.0	12.8	8.5
Breakfast cereals	1.1	0.5	0.2	0.0	0.0	0.0	1.1	0.4
Cereal-based dishes	0.8	1.4	0.7	0.0	0.0	0.1	0.8	1.2
Pasta, rice, and other cereals	1.6	1.3	0.6	0.4	0.0	0.2	1.6	1.2
Total cereal products	16.4	13.1	1.6	0.4	0.0	0.3	16.3	11.3
Butter	0.3	0.9	1.2	0.0	0.0	0.0	0.3	0.8
Cheese	1.2	4.4	4.3	0.0	0.0	0.0	1.3	3.7
Cream	0.1	0.5	0.0	0.0	0.0	0.0	0.1	0.4
Milk	0.2	0.6	0.4	0.0	0.0	0.0	0.2	0.6
Yogurts	0.1	0.2	0.0	0.0	0.0	0.0	0.1	0.2
Total dairy products	1.9	6.6	5.9	0.0	0.0	0.0	2.0	5.6
Fatty sauces	11.3	25.1	0.0	0.1	0.1	0.0	11.2	21.6
Margarines	15.7	17.7	0.0	0.9	0.0	0.5	15.6	15.3
Mixed fats	0.1	0.2	0.0	0.0	0.0	0.0	0.1	0.1
Vegetable oils	4.3	1.8	0.0	0.0	0.0	0.0	4.3	1.6
Total fats and oils	31.4	44.7	0.0	1.1	0.1	0.5	31.2	38.6
Fatty fish	0.4	0.6	5.0	47.4	49.8	48.1	0.4	7.3
Fish products	0.4	0.1	0.4	3.2	1.9	3.0	0.4	0.5
Half-fatty fish	0.0	0.0	1.2	5.2	3.8	6.6	0.0	0.8
Lean fish	0.0	0.0	2.2	7.6	4.5	9.5	0.0	1.2
Molluscs and crustaceans	0.0	0.1	8.9	23.9	6.1	12.9	0.1	2.3
Total fish and seafood	0.8	0.9	17.8	87.3	66.0	80.0	0.9	12.1
Fruits	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.4
Legumes	0.1	0.3	0.0	0.0	0.0	0.0	0.1	0.3
Nuts and seeds	6.6	3.5	0.0	0.6	0.0	0.0	6.6	3.1
Potatoes	5.5	1.7	0.0	0.0	0.0	0.0	5.5	1.5
Soups	0.6	0.6	0.8	0.1	0.0	0.2	0.6	0.5
Vegetables	0.6	1.8	0.0	0.1	0.0	0.0	0.6	1.5
Total fruits and vegetables	13.4	8.4	0.8	0.8	0.0	0.2	13.4	7.2
Eggs	1.1	0.3	25.5	0.2	6.2	6.1	1.2	0.9
Meat and meat dishes	8.4	8.3	17.9	2.4	12.1	2.0	8.5	7.6
Poultry	1.6	1.2	15.3	2.7	12.9	3.7	1.6	1.7
Total meat, poultry, and eggs	11.1	9.9	58.7	5.2	31.2	11.8	11.3	10.1
Biscuits	2.9	2.7	0.2	0.0	0.0	0.0	2.8	2.4
Chocolate products	5.4	3.7	0.0	0.0	0.0	0.0	5.4	3.2
Pastry and desserts	4.5	4.4	8.7	0.1	0.1	0.9	4.5	3.8
Sugar and sweets	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Total sweet products	12.8	10.8	9.0	0.1	0.1	0.9	12.8	9.3
Dietetic products	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Miscellaneous	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Salty snacks	2.2	0.7	0.0	0.0	0.2	0.0	2.1	0.6
Snacks	6.8	2.9	6.2	4.3	2.3	6.2	6.8	3.2
Soydrink	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Spices and condiments	0.5	0.8	0.0	0.0	0.0	0.0	0.5	0.7
Vegetarian substitute	2.6	1.3	0.0	0.8	0.0	0.0	2.6	1.2
Total miscellaneous	12.1	5.8	6.2	5.1	2.5	6.2	12.1	5.7

[a]Σn-6 = C18:2n-6 + C20:4n-6.
[b]Σn-3 = C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3.

of the overall intake of EPA, DPA, and DHA. For EPA, the subgroup molluscs and crustacean were also quite important (accounting for 23.9%). A substantial part of the DPA intake was contributed by poultry (13.0%), meat and meat dishes (12.1%), and eggs (6.1%). This group contributed also to the DHA intake (for 11.8%), with eggs as the major subgroup (6.1%). Since fortification of margarines with LC n-3 PUFA and since the use of eggs in pastry and desserts, as well as in certain snacks, these food items also contribute a bit to the intake of LC n-3 PUFA intake.

DISCUSSION

Some methodological shortcomings related to this study have to be mentioned. First, it has to be said that extreme intakes (low and high) are likely to be exaggerated because the consumption data were derived only from a 2-d food record. Two days are not enough to give a picture of the usual intake on an individual level, especially for nutrients abundantly present in foods that are not consumed on a daily basis, such as seafood products. There is a high within- and between-subject variance

TABLE 7
Fat and PUFA Intake Data of Different Other Larger and Smaller Population Studies of Women

Country	Reference	Population	Method	Fat	LA	LNA	AA	EPA	DPA	DHA	Σn-6[a]	Σn-3[b]	LA/LNA	n-6/n-3
					g/d		mg/d				g/d			
Larger sample														
Belgium	This study	641 women (18–39 y)	2-day record	77.58	11.62	1.44	55.90	77.80	25.30	131.20	11.97	1.67	8.70	7.8
Australia	Howe et al. 2005	5,770 women (19+ y)	24-h recall	NA[c]	8.72	0.87	117.00	60.00	52.00	83.00	8.93	1.06	NA	NA
France	Astorg et al. 2004	2,785 women (35–63 y)	6 × 24-h record	73.55	8.10	0.74	151.90	117.80	55.90	225.90	NA	NA	11.10	NA
Germany	Linseinen et al. 2003	898 women (35–64 y)	24-h recall	76.10	11.60	1.51	140.00	80.00	NA	140.00	11.70	1.72	NA	7.21
Germany	Linseinen et al. 2003	1,078 women (35–64 y)	24-h recall	78.00	10.90	1.32	160.00	70.00	NA	140.00	11.00	1.53	NA	7.99
Australia	Meyer et al. 2003	3,178 women (19–64 y)	24- h record	NA	9.40	1.02	41.00	46.00	21.00	89.00	9.40	1.17	8	NA
Japan	Tokudome et al. 1999	180 women (middle-aged)	1-d record	57.7	11.21	1.71	139	242.00	NA	469.00	NA	NA	NA	NA
Norway	Johansson et al. 1998	1,627 women (16–79 y)	FFQ	67.00	8.80	1.20	120.00	270.00	60.00	400.00	NA	NA	NA	4.17
Smaller sample														
United States	Loosemore et al. 2004	31 women (mean: 25 y)	2 × 24-h recall	74.03	11.56	1.34	155.38	15.81	NA	67.69	11.71	1.46	9.04	4.53
Japan	Tokudome et al. 2003	71 women (40–49 y)	7-d record	NA	10.48	1.57	135.25	278.25	74.75	496.75	10.6	2.4	NA	4.63
Canada	Innis et al. 2003	55 pregnant women (20–40 y)	FFQ	79.80	11.20	1.60	121.00	78.00	NA	160.00	NA	NA	NA	NA
Belgium	De Vriese et al. 2001	26 pregnant women; 1st trimester	FFQ	85.90	12.90	1.30	130.00	170.00	NA	300.00	13.20	1.82	NA	NA
Belgium	De Vriese et al. 2001	26 pregnant women; 3rd trimester	FFQ	90.20	13.70	1.50	130.00	150.00	NA	300.00	14.00	1.98	NA	NA
Netherlands	Otto et al. 2001	19 pre-pregnant women (mean: 30.7 y)	FFQ	88.20	13.22	1.09	30.00	50.00	10.00	90.00	13.24	1.27	NA	11.31
Netherlands	Otto et al. 2001	20 pregnant women (mean: 30.7 y)	FFQ	86.94	13.17	1.02	20.00	80.00	20.00	140.00	13.19	1.29	NA	11.97

[a]Σ n-6 = 18:2n-6 + 20:4n-6; except for Meyer *et al.* (16) Σ n-6 = 18:2n-6 + 20:2n-6 + 20:3n-6 + 20:4n-6 + 22:4n-6; Tokudome *et al.* (12) = 18:2n-6 + 18:3n-6 + 20:3n-6 + 20:4n-6; Otto *et al.* (31) Σ n-6 = 18:2n-6 + 20:3n-6 + 20:4n-6 + 22:4n-6 + 22:5n-6.

[b]Σ n-3 = 18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3; except for Linseinen *et al.* (17) and De Vriese *et al.* (31) Σ n-3 = 18:3n-3 + 20:5n-3 + 22:6n-3.

[c]NA, not available. For other abbreviations, see Table 1.

for these nutrient intakes over different days. Nelson *et al.* (28) calculated the days needed to assess the individual PUFA intake with a good correlation ($r \geq 0.9$) between the observed and the true mean intake. For women older than 18 y, at least 30 d should be necessary. Nevertheless, the 2-d food record yields unbiased estimates of the mean food consumption of a population sample.

Second, at the level of the food composition data, a point of discussion is that PUFA, in particular the long-chain PUFA, are present in only small amounts in individual foods, but accumulate to significant levels of biological importance in the context of a whole diet. For most foods, these low values often round down to zero when reported to a single decimal place, and so it is likely that FA concentrations will be consistently underreported in FCDB (29). Furthermore, for some items it was not easy to find the FA composition. Quinoa, for example, was only mentioned in the German FCDB (26). We were obliged to use eight different databases in order to determine the FA composition of all food items, which is not an ideal situation. Moreover, a very recent Australian study (11) noted that the meat and poultry category of foods was a significant source of LC n-3 PUFA. The authors stated that the modest amount of PUFA in muscle tissue phospholipids of lean meat needs to be taken into account when determining dietary PUFA intakes. The majority of LC n-3 PUFA in meat is DPA. According to Howe *et al.* (11), the LC n-3 PUFA content of meat products has been underestimated up to now. Because of the limitations of available data about the FA content of these food items, LC n-3 intake from meat, poultry, and eggs, especially DPA intake, could have been underestimated, but this is not likely to have resulted in a significant underestimation of the mean EPA and DHA intake, given that these two FA are mainly brought by fish and seafood. Moreover, the Belgian recommendation considers only EPA and DHA for the evaluation of LC n-3 PUFA intake. Therefore, the evaluation of the intake data against the Belgian recommendations is not influenced by the possible underestimation of DPA during the intake assessment.

The recommendations formulated by the Belgian Health Council regarding fat and FA (Table 1), were used to evaluate the intakes of this study population. It is recommended that the total fat intake not exceed 30% of E per day. Three-quarters of the population exceeded the tolerable upper intake limit for total fat (30%E) and SFA (10%E), whereas MUFA and PUFA corresponded to the recommendations. In contrast to the intakes of LA and ∑n-6 PUFA, which fit the recommendations for the major part of the population, the mean and median intakes of LNA and ∑n-3 PUFA fell well below the recommended intake (1%E for LNA). The same is true for LC n-3 PUFA. The recommended minimum is expressed for the sum of EPA and DHA, and is at least equal to 0.3%E. The mean intake for EPA + DHA was 0.10%E, and the median was 0.20%E. It can be concluded that the study population has an important deficit for these FA.

Published data of mean dietary intake of PUFA in adult women, expressed in g/d or mg/d, are presented in Table 7.

Nevertheless, the comparisons must be interpreted with caution as survey methods can vary. Moreover, non-negligible differences exist in the age and the total energy intake of the population.

The women of the studies in France, Norway, and Australia seemed to consume less LA, whereas the women in Germany and Japan consumed quite similar levels (11,12,14–17). Conversely, higher absolute LA intake levels were found in another Belgian and a Dutch study with a smaller sample size (30,31) (Table 7). Another pattern was found for LNA. The absolute intake of LNA determined in this study is quite high when compared with other studies, with exception of the Japanese and German studies (12,17) and a study done in pregnant Canadian women (32). In all the other studies, a lower LNA intake was determined. This is remarkable, because even in the present study a comparison of the LNA intake with the Belgian recommendations showed a deficit of this FA in the diet of the Belgian women. Astorg *et al.* (14) translated the mean LNA intake of the female population group in their study into %E; it was 0.38%E. Comparing this with the Belgian recommended minimum level, the French women have an even worse deficit than the Belgian women. Correspondingly, the mean LA/LNA ratio of the French women is far higher (11.10) than the one found in this study. Whereas the essential n-6 PUFA intake seemed rather high in comparison with other studies, the opposite applies to the mean absolute intake of AA, the only LC n-6 PUFA taken into consideration in this study. Most of the other studies found a mean absolute AA intake that was two to three times higher than the mean AA intake determined in this study. Nevertheless, the intake of the food sources that contribute the most AA to the diet are equal compared with those found in the French and the Australian studies (14,16); they are (1) meat, poultry, and eggs (accounting for more than 50% of the AA intake), and (2) fish and seafood. So a probable explanation is that the AA concentration of these food items is underestimated in the FCDBs that were used in this study.

A large variation is found in the intake estimations of LC n-3 PUFA between studies and countries. As for LA and LNA mean intake, the mean LC n-3 PUFA intake is comparable with the results found in the German study (17), as well as with the results of the Dutch study (31). A larger absolute intake of EPA, DPA, and DHA is found in France and especially in Norway and Japan, probably due to larger seafood consumption. In this study, the average seafood consumption of the women (calculated as a mean of the 2 d) was 27.0 (± 44.9) g/d. Welch *et al.* (33) reported the seafood consumption in 10 European countries for men and women between 35 and 74 years old. For women, a mean seafood consumption of 15.9 g/d and 19.9 g/d was found in two German cities, and a mean of 13.3 g/d and 13.4 g/d in two Dutch cities (33). In comparison, French women have a mean seafood consumption ranging between 35.0 g/d and 52.4 g/d, depending on the region where they live; the mean intake of seafood for Norwegian women was 42.9 g/d in south and east Norway and 63.3 g/d in north and west Norway (33). Johansson *et al.* (15) reported a mean intake of 57 g of fish per day for Norwegian women. These results show that the higher seafood consumption in the latter two countries is clearly the main reason for the higher LC n-3 PUFA intake.

Comparisons of food sources of fat and FA are not easy, because the definitions of food groups are not the same in the different studies. The definition of the food groups in this study is based on the French study of Astorg *et al.* (14), so the results of these two studies are quite comparable. The comparison with other results must be seen as an indication.

The main sources for LA found in this study were fats and oils (31.4%), followed by cereal products (16.4%). The important contribution of fats and oils to the LA intake was also found in the French and the Australian studies (accounting for 33.5% and 21.9%, respectively) (14,16), as well as in the Japanese study (13). For AA, the intake was mostly contributed by meat, poultry, and eggs, followed by fish and seafood. The same results were found by the French and Australian authors: meat, poultry, and eggs accounted for 67.2% and 70.15%, respectively, followed by fish and seafood (11.1% and 27.2%, respectively). In the Japanese study, chicken, eggs, and pork together contributed 50.6% to the AA intake.

In the present study, fats and oils were by far the major contributor to LNA intake (45%), especially fatty sauces and margarines. This is at variance with the French study, where fats and oils were only a minor contributor (10%), the major ones being dairy products, fruits and vegetables, and meat, poultry, and eggs (14). Fatty sauces and margarines seem to be much less consumed in France than in Belgium.

In this study, fish and seafood were the major contributors to the EPA, DPA, and DHA intake, representing respectively 87.3%, 66.0%, and 80.0%. In Norway, fish was also the major source (respectively, accounting for 55%, 43%, and 57%), followed by cod liver oil, which was used by 36% of the survey subjects (15). In contrast, cod liver oil, being rich in LC n-3 PUFA, was not consumed by the Belgian women included in this study. In the French data, fish and seafood were the major sources for EPA and DHA (72.0% and 64.7%, respectively) (14), but meat, poultry, and eggs were the first contributor of DPA intake (55%). In Norway, meat and fish were the main sources of DPA (43% each) (15). In the present study, the contribution of meat, poultry, and eggs to the DPA intake was lower (31%). A very recent Australian publication described in detail the contribution of meat sources to the intake of LC n-3 PUFA (11). Their analyses showed that fish and seafood contributed 49.7%, 15.4%, and 69.9% to the intake of, respectively, EPA, DPA, and DHA. On the other hand, meat, poultry, and game products provided, respectively, 44.8%, 73.2%, and 19.6%. This shows that an underestimation of the LC n-3 PUFA content of meat can be crucial, since the consumption of meat and meat products is large compared with that of fish and seafood.

CONCLUSION

In conclusion, analysis showed that the mean intake of total fat and SFA of Belgian women exceeded the Belgian recommendations. With regard to the PUFA intake, the mean intake of n-6

PUFA seemed to be sufficient. In contrast, the mean intakes of LNA and LC n-3 PUFA were far too low. When considering these facts together, it seems advisable to explore ways to investigate food-based strategies to decrease the SFA intake and also increase the n-3 PUFA intake; this is a counterargument to the intake of supplements. Although such supplements can be an easy solution for some women, the presence of other nutrients in food sources of EPA and DHA, such as important amino acids and trace elements, must also be considered. This conclusion is in accordance with one of the conclusions in a recent Dutch report, which stated that an excessive intake of the unfavorable types of fat, such as SFA, increases the likelihood of developing cardiovascular disease by 25%, whereas eating fish once or twice a week reduces this risk 25% (34).

A previous study in Belgium of consumer awareness and beliefs related to n-3 PUFA, their health benefits, and their food sources showed that awareness is poor and beliefs often wrong (35). Nutrition education should play an important role in convincing people of this dietary shift.

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Influence of Dietary Oil Content and Conjugated Linoleic Acid (CLA) on Lipid Metabolism Enzyme Activities and Gene Expression in Tissues of Atlantic Salmon (*Salmo salar* L.)

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ABSTRACT: The overall objective is to test the hypothesis that conjugated linoleic acid (CLA) has beneficial effects in Atlantic salmon as a result of affecting lipid and FA metabolism. The specific aims of the present study were to determine the effects of CLA on some key pathways of FA metabolism, including FA oxidation and highly unsaturated FA (HUFA) synthesis. Salmon smolts were fed diets containing two levels of fish oil (low, ~17%, and high, ~34%) containing three levels of CLA (a 1:1 mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 at 0, 1, and 2% of diet) for 3 mon. The effects of dietary CLA on HUFA synthesis and β-oxidation were measured, and the expression of key genes in the FA oxidation and HUFA synthesis pathways, and the potentially important transcription factors peroxisome proliferators activated receptors (PPAR), were determined in selected tissues. Liver HUFA synthesis and desaturase gene expression was increased by dietary CLA and decreased by high dietary oil content. Carnitine palmitoyltransferase-I (CPT-I) activity and gene expression were generally increased by CLA in muscle tissues although they were relatively unaffected by dietary oil content. In general CPT-I activity or gene expression was not correlated with β-oxidation. Dietary CLA tended to increase PPARα and β gene expression in both liver and muscle tissues, and PPARγ in liver. In summary, gene expression and activity of the FA pathways were altered in response to dietary CLA and/or oil content, with data suggesting that PPAR are also regulated in response to CLA. Correlations were observed between dietary CLA, liver HUFA synthesis and desaturase gene expression, and liver PPARα expression, and also between dietary CLA, CPT-I expression and activity, and PPARα expression in muscle tissues. In conclusion, this study suggests that dietary CLA has effects on FA metabolism in Atlantic salmon and on PPAR transcription factors. However, further work is required to assess the potential of CLA as a dietary supplement, and the role of PPAR in the regulation of lipid metabolism in fish.

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Abbreviations: CPT-I, carnitine palmitoyl transferase-I; HUFA, highly unsaturated FA; PPAR, peroxisome proliferator-activated receptor; rtqPCR, real-time quantitative polymerase chain reaction; SCD, stearoyl CoA desaturase; SGR, specific growth rate; SREBP, steryl regulatory element binding protein.

Conjugated linoleic acid (CLA) refers to conjugated isomers of linoleic acid (18:2n-6); the two main naturally occurring isomers are *cis*-9,*trans*-11 and *trans*-10,*cis*-12. In mammals, dietary CLA has been shown to have a number of physiological effects and health benefits, including anticarcinogenic and immune enhancing properties (1). In addition, CLA has several beneficial effects on lipid metabolism in mammals, including altering body composition (2). Specifically, CLA has been shown to decrease body fat and increase lean body mass in mice, rats, and pigs (2). Decreased body fat has also been observed in human studies, although the effect was much less than that observed with mice (3). CLA is also known to decrease the activity and gene expression of mammalian stearoyl CoA Δ9 desaturase (4), and may also suppress Δ6 and Δ5 desaturase (5) and elongase (6).

There is accumulating evidence that some of the effects of CLA may be mediated through transcription factors such as peroxisome proliferator activated receptors (PPAR) or sterol regulatory element binding proteins (SREBP). Specifically CLA isomers are ligands and activators of both PPARα (7) and PPARγ (8), which in mammals are known to regulate the expression of genes of FA oxidation and lipid deposition in liver and adipose tissue, respectively (9). Dietary CLA also influenced the expression of SREBPs in ob/ob mice (10). Both PPARs and SREBPs are involved in the control of FA desaturation and elongation, with PPARα and SREBP-1 both affecting Δ6 and Δ5 activities (11), and SREBP-1 also regulating elongase activities (12). Therefore, CLA via PPARs and/or SREBPs may affect lipid contents and FA compositions in a variety of ways including selectivity in oxidation and deposition of specific FA and modulation of FA desaturation and elongation.

The overall objective is to test the hypothesis that CLA has beneficial effects in Atlantic salmon as a result of affecting lipid and FA metabolism. The specific aims of the present study were to determine the effects of CLA on some key pathways of FA metabolism, including FA oxidation and highly unsaturated FA (HUFA) synthesis via desaturation and elongation. To this end, salmon smolts were fed diets containing two levels of fish oil (low, ~17%, and high, ~34%) containing three levels of CLA (a 1:1 mixture of the two main isomers

present in nature, *cis*-9,*trans*-11 and *trans*-10,*cis*-12, at 0, 1, and 2% of diet) for 3 mon. The effects of dietary CLA on the expression of key genes of FA oxidation and HUFA synthesis, and on the enzymatic activities of the pathways were determined. In addition, the effect of CLA on the expression of potentially important transcription factors, PPAR, was determined in selected tissues.

EXPERIMENTAL PROCEDURES

Diets and animals. Atlantic salmon smolts (S1/2) were obtained from a commercial salmonid farm (Howietoun Fish Farm, Sauchieburn, Scotland) in late October and transported to Stirling University, Institute of Aquaculture, Marine Environmental Research Laboratory, Machrihanish, Scotland. The fish were maintained in stock tanks for 3 wk at ambient water temperature of about 10–11°C to acclimatize, during which time the fish were fed standard salmon diet, before being randomly distributed between 18 indoor, round, conical tanks of 1.5 m^3 volume (1.72 m diameter). The initial stocking density was 100 fish of average fish weight 87.5 ± 1.6 g per tank (5.8 kg/m^3). Water temperature was maintained at 12 ± 1°C throughout the trial, with a light regime of 12 h light:12 h dark. Six experimental diets were fed to triplicate tanks for 3 mon, with feed supplied to appetite manually. The experimental diets were formulated to satisfy the nutritional requirements of salmonid fish (13), and were formulated and manufactured by BioMar A/S (Brande, Denmark). The trial had a 3×2 factorial design with CLA added at three concentrations to diets with two oil contents (low and high). Thus diets were produced with 0, 1, and 2% CLA replacing standard Northern hemisphere fish oil in smolt feeds containing either 17 or 34% total lipid. The low-oil diets contained 17% total lipid with 0 (L0), 1 (L1), and 2% (L2) CLA. The high-oil diets, labeled H0, H1, and H2, contained 34% total lipid. Diets were formulated to be isonitrogenous; protein content was constant between diets of different oil content (Table 1). Diets within the low or high oil groups were identical in formulation other than FA composition, with CLA (LUTA-CLA™ 60, containing 60% CLA methyl esters as a 50:50 mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers; BASF AG, Ludwigshafen, Germany) balanced by fish oil (capelin oil, Norsemeal Ltd., London, United Kingdom). The FA compositions of the diets are presented in Table 2.

Sampling protocols. At the termination of the trial, 10 fish per dietary treatment (3–4/tank) were individually weighed and eviscerated, and hepato- and viscero-somatic indices were determined. Samples of 0.5 g of liver, white muscle, and red muscle were rapidly dissected from six of the fish (2/tank) and immediately frozen in liquid nitrogen for RNA analyses. In addition, samples of 1–2 g of liver, white muscle, and red muscle for biochemical analyses were collected from the same fish and immediately frozen in liquid nitrogen. All samples were subsequently stored at –80°C prior to analysis.

RNA extraction and real-time quantitative polymerase chain reaction (rtqPCR). Total RNA was isolated from 100–500 μg (depending upon tissue) of pooled (2 fish) tissue by the standard TRIzol method (Invitrogen Ltd, Paisley, United Kingdom), and DNA was removed using DNA-*free*™ DNase treatment (Ambion Inc., Austin, TX). Total RNA (5 μg) was reverse transcribed into cDNA using M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega UK, Southampton, United Kingdom). The PCR primers were designed according to the salmon cDNA sequences for Δ6 desaturase (accession no. AY458652), Δ5 desaturase (accession no. AF478472), CPT-I, PPARα (M.J. Leaver, personal communication), PPARβ (accession no. AJ416953), and PPARγ (accession no. AJ416951). Primer sequences and PCR product sizes are given in Table 3. The linearized plasmid DNA containing the target sequence for each gene was quantified to generate a standard curve of known copy number. Amplification of cDNA samples and DNA standards was carried out using the SYBR Green PCR Kit (Applied Biosystems, Foster City, CA) with the following conditions: denaturation for 15

TABLE 1
Formulations (Percentage of Dry Ingredients) and Proximate Compositions (Percentage of Total Diet) of Experimental Diets

	L0	L1	L2	H0	H1	H2
Fishmeal	44	44	44	50	50	50
Sunflower meal	15	15	15	4	4	4
Corn gluten	8	8	8	8	8	8
Legume seeds	9	9	9	9	9	9
Cereal grains	10	10	10	0	0	0
Micronutrients	3	3	3	2	2	2
Fish oil	11.0	9.3	7.6	27.0	25.3	23.6
CLA	0	1.7	3.4	0	1.7	3.4
Moisture	8.0 ± 0.3	8.7 ± 0.1	8.9 ± 0.1	3.1 ± 0.1	4.2 ± 0.4	4.4 ± 0.1
Lipid	18.2 ± 0.3	17.4 ± 0.1	16.4 ± 0.5	33.2 ± 0.6	32.4 ± 1.2	32.6 ± 1.6
Protein	44.8 ± 0.1	44.8 ± 0.2	45.8 ± 0.5	47.0 ± 0.3	47.0 ± 0.3	47.1 ± 1.3
Ash	7.8 ± 0.1	7.8 ± 0.1	7.9 ± 0.1	8.2 ± 0.1	8.1 ± 0.0	8.0 ± 0.2

Results for proximate compositions are means ± SD (n = 3). Micronutrients include essential amino acids (methionine and lysine), vitamins, minerals, and astaxanthin (Carophyll pink®). H0, H1, and H2, diets containing fish oil at 34% and supplemented with 0, 1, and 2% CLA; L0, L1, and L2, diets containing fish oil at 18% and supplemented with 0, 1, and 2% CLA.

TABLE 2
Fatty Acid Composition (Percentage of Weight) of Experimental Diets Containing CLA Fed to Atlantic Salmon (*Salmo salar*).

	L0	L1	L2	H0	H1	H2
Total saturated	30.7 ± 1.0^{ab}	28.6 ± 1.1^{b}	28.9 ± 1.4^{b}	31.9 ± 0.4^{a}	30.6 ± 0.7^{ab}	31.0 ± 0.8^{ab}
Total monoenes	30.1 ± 0.7	30.0 ± 0.7	31.0 ± 0.9	29.8 ± 0.6	29.3 ± 0.4	30.2 ± 0.5
18:2n-6	6.4 ± 0.2^{b}	6.6 ± 0.1^{ab}	6.8 ± 0.2^{a}	3.1 ± 0.1^{c}	3.3 ± 0.0^{c}	3.2 ± 0.0^{c}
20:4n-6	0.9 ± 0.0^{ab}	0.8 ± 0.0^{bc}	0.7 ± 0.0^{c}	1.0 ± 0.1^{a}	0.9 ± 0.0^{ab}	0.8 ± 0.0^{bc}
Total n-6 PUFA[a]	8.4 ± 0.1^{a}	8.2 ± 0.5^{a}	8.2 ± 0.6^{a}	5.1 ± 0.1^{b}	5.0 ± 0.2^{b}	4.8 ± 0.5^{b}
18:3n-3	1.2 ± 0.0^{a}	1.1 ± 0.0^{ab}	1.0 ± 0.0^{a}	1.2 ± 0.0^{a}	1.2 ± 0.0^{a}	1.1 ± 0.0^{ab}
18:4n-3	2.5 ± 0.1^{b}	2.1 ± 0.0^{d}	1.8 ± 0.0^{c}	2.7 ± 0.0^{a}	2.6 ± 0.0^{ab}	2.3 ± 0.0^{c}
20:4n-3	0.7 ± 0.0^{ab}	0.6 ± 0.0^{bc}	0.5 ± 0.0^{c}	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}	0.7 ± 0.0^{ab}
20:5n-3	11.8 ± 0.7^{b}	10.2 ± 0.2^{c}	8.4 ± 0.5^{d}	13.6 ± 0.1^{a}	13.0 ± 0.1^{a}	11.5 ± 0.1^{b}
22:5n-3	1.8 ± 0.1^{b}	1.6 ± 0.0^{c}	1.3 ± 0.1^{d}	2.0 ± 0.0^{a}	1.9 ± 0.0^{a}	1.7 ± 0.0^{bc}
22:6n-3	12.7 ± 0.9^{a}	11.5 ± 0.5^{b}	9.5 ± 0.1^{c}	12.9 ± 0.0^{a}	12.4 ± 0.2^{a}	10.9 ± 0.1^{b}
Total n-3 PUFA[b]	30.8 ± 1.8^{a}	27.3 ± 0.8^{b}	22.6 ± 0.7^{c}	33.2 ± 0.3^{a}	31.8 ± 0.4^{a}	28.2 ± 0.2^{b}
CLA (*cis*-9,*trans*-11)	0.0 ± 0.0^{d}	2.9 ± 0.5^{b}	4.8 ± 1.6^{a}	0.0 ± 0.0^{d}	1.6 ± 0.2^{c}	2.9 ± 0.7^{b}
CLA (*trans*-10,*cis*-12)	0.0 ± 0.0^{d}	3.0 ± 0.4^{b}	4.6 ± 1.7^{a}	0.0 ± 0.0^{d}	1.7 ± 0.4^{c}	2.9 ± 0.8^{b}
Total CLA	0.0 ± 0.0^{d}	5.9 ± 0.5^{b}	9.4 ± 1.5^{a}	0.0 ± 0.0^{d}	3.3 ± 0.4^{c}	5.8 ± 0.7^{b}
Total PUFA	39.2 ± 1.7	41.5 ± 1.8	40.2 ± 2.4	38.3 ± 0.2	40.1 ± 1.1	38.8 ± 1.3

Values are means ± SD of three samples. Significance of differences between mean values were determined by one-way ANOVA followed, where appropriate, by Tukey's multiple comparison test as described in the text. [a–d]Values within a row with a different superscript letter are significantly different ($P < 0.05$). [a]Totals include 18:3n-6, 20:2n-6, and 22:5n-6 present in some samples at up to 0.4%. [b]Totals contain 20:3n-3 present at up to 0.2%. H0, H1, and H2, diets containing fish oil at 34% and supplemented with 0, 1, and 2% CLA; L0, L1, and L2, diets containing fish oil at 18% and supplemented with 0, 1, and 2% CLA.

TABLE 3
Forward (Sense) and Reverse (Antisense) Primers used for Real-Time Quantitative PCR

Gene	PCR product length	Forward primer	Reverse primer
Δ5 Desaturase	192	5′-GTGAATGGGGATCCATAGCA-3′	5′-AAACGAACGGACAACCAGA-3′
Δ6 Desaturase	181	5′-CCCCAGACGTTTGTGTCAG-3′	5′-CCTGGATTGTTGCTTTGGAT-3′
CPT-I	161	5′-GAGAGAGCTGCGACTGAAAC-3′	5′-GACAGCACCTCTTTGAGGAA-3′
PPARα	204	5′-ATCTTTCCACTGCTGCCAGTGC-3′	5′-GATGAAGCCCGATCCGTAGGCCACCAGG-3′
PPARβ1	517	5′-TACCGCTGCCAGTGCACCACGGTG-3′	5′-TTCTGGACCAAGCTGGCGTTCTCA-3′
PPARγ	266	5′-TATCTCCCCTCTCTAGAGTA-3′	5′-AGGGCTTATCGTTTACTGAACCTTGATACACGC-3′

min at 95°C followed by 45 cycles of 15 s at 95°C, 15 s at 56°C, and 30 s at 72°C, followed by product melting to check purity of PCR product. Thermal cycling and fluorescence detection were conducted using the Rotor-Gene 3000 system (Corbett Research, Cambridge, United Kingdom). Expression of target genes was normalized using a Quant-iT™ high-sensitivity DNA assay kit containing PicoGreen reagent, (Molecular Probes, Rijinsburgerweg, the Netherlands) using a modified version of the manufacturer's protocol (14). All reactions were carried out in duplicate and a linear standard curve was drawn, plotting the average absorbance values obtained for the Quant-iT DNA values against the standard DNA concentration, with the concentration of total cDNA calculated from the linear equation.

FA desaturation and elongation (HUFA synthesis) in liver microsomes. Portions of frozen liver were homogenized to 10% (w/v) in 0.25 M sucrose in 40 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 0.15 M KCl, 40 mM KF, and 1 mM *N*-acetyl cysteine. The homogenate was centrifuged at 25,000*g* for 15 min at 4°C, the floating fat layer removed by aspiration, and the remaining supernatant centrifuged at 105,000*g* for 60 min at 4°C. The floating fat layer was aspirated and the supernatant decanted. The pelleted microsomal fraction was resuspended in 1 mL of sucrose buffer, and 50 μL was taken for protein determination following the procedure of Lowry *et al.* (15) after incubation with 0.45 mL of 0.25% (w/v) SDS/1 M NaOH for 45 min at 60°C. The assay mixture, in sucrose buffer (pH 7.4), contained 5 mM $MgCl_2$, 1.5 mM glutathione, 0.5 mM nicotinamide, 1 mM NADH, 100 μM coenzyme A, and 5 mM ATP in a total volume of 0.75 mL. Fifty μL of [1-^{14}C]18:3n-3 (0.25 μCi, 5 μM final concentration), as a fatty acid-free (FAF) BSA complex, was added and the reaction initiated by the addition of 200 μL of microsomes. Incubation continued for 1 h at 20°C, and the reaction was stopped by the addition of 5 mL of chloroform/methanol (2:1, vol/vol) containing 0.01% BHT, and total lipid was extracted, transmethylated, and FAME prepared as described previously (16). FAME were separated by argentation TLC and visualized by autoradiography; radioactivity was determined by scintillation counting as described previously (17).

Assay of carnitine palmitoyltransferase I (CPT-I) and FA β-oxidation in tissue homogenates. Liver, red muscle, and white muscle were weighed, diced, and homogenized to 20%

(w/v) in 0.25 M sucrose in 10 mM HEPES buffer and 1 mM EDTA at pH 7.4. The homogenates were centrifuged at 1880*g* for 10 min at 4°C, the floating fat layer was aspirated, the postnuclear fractions were collected, and 50 μL was taken for protein determination as above. Portions were used immediately for determination of enzyme activities. CPT-I activity was estimated by determining the production of palmitoyl[^{3}H]carnitine from palmitoyl CoA and [^{3}H]carnitine essentially as described by Saggerson and Carpenter (18). FA β-oxidation was estimated by determining radioactivity in acid-soluble products derived from the oxidation of [1-^{14}C]palmitoyl-CoA as described by Torstensen *et al.* (19).

Materials. [1-^{14}C] Palmitoyl CoA (50–55 mCi/mmol) and [methyl-^{3}H] L-carnitine hydrochloride (60–86 Ci/mmol) were obtained from GE Healthcare Bio-Sciences (Little Chalfont, Bucks, United Kingdom), and [1-^{14}C]18:3n-3 (50–55 mCi/mmol) was obtained from NEN (PerkinElmer LAS UK Ltd., Beaconsfield, United Kingdom). BHT, carnitine, FAF-BSA, HEPES, palmitoyl-CoA, silver nitrate, and TriReagent were obtained from Sigma Chemical Co. (Poole, United Kingdom). TLC (20 cm × 20 cm × 0.25 mm) plates, precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Fisher Scientific UK (Loughborough, United Kingdom).

Statistical analysis. All data are presented as means ± SD (*n* value as stated). Percentage data and data that were identified as nonhomogeneous (using Bartlett's test) were subjected to arcsine transformation before analysis. The effects of dietary CLA and oil content were determined by two-way ANOVA with Bonferroni posttests to determine significance of differences due to CLA. Differences were regarded as significant when $P < 0.05$ (20).

RESULTS

Diet composition, growth, and biometry. Inclusion of CLA in the low-oil diets resulted in levels of total CLA of 5.9 and 9.5% of total FA at the 1 and 2% inclusion levels, respectively (Table 2). Obviously, in the high-oil diets, inclusion of CLA at 1 and 2% resulted in lower levels of total CLA, at 3.3 and 5.8% of total FA, respectively. The levels of CLA in relative terms were identical in the L1 and H2 diets, with an overall rank order for CLA content of L2 > L1 = H2 > H1 > L0 = H0. There were no effects of diet on growth parameters, with no significant effects of CLA or oil content on final weights or specific growth rate (SGR) (Table 4).

Tissue enzyme activities. HUFA synthesis in liver microsomes, as measured by the recovery of radioactivity in the summed Δ6 and Δ5 desaturated products (18:4, 20:4, 20:5, and 22:5) of [1-^{14}C]18:3n-3, was significantly affected by both dietary oil content and CLA content as determined by two-way ANOVA (Table 5). However, there was significant interaction between the variables, as activity was significantly higher in fish fed 2% CLA compared to fish fed fish oil alone at the low-oil level, whereas the opposite trend was observed at the high-oil level (Fig. 1). HUFA synthesis activity in liver was significantly lower in fish fed the high-oil diet (Table 5, Fig. 1).

Dietary CLA did not significantly increase β-oxidation activity in white muscle irrespective of dietary oil content (Table 5, Fig. 2). There was a trend suggesting CLA may increase β-oxidation in red muscle in fish fed high dietary oil, but the high variation made this nonsignificant. Liver β-oxidation was significantly lower in fish fed the high-oil diet, but dietary CLA had no effect on β-oxidation in liver (Table 5, Fig. 2). Dietary oil content had no effect on β-oxidation activities in either muscle tissue.

CPT-I activity was significantly increased by dietary CLA in white muscle, and also in red muscle, particularly in fish fed the high-oil diets (Table 5, Fig. 3). CPT-I activity in white

TABLE 4
Final Weights of Atlantic Salmon (*Salmo salar*) Fed Diets Containing CLA

Diet	Initial weight (g)	Final weights (g)	SGR
L0	87.8 ± 1.5	256.3 ± 18.7	1.31
L1	87.4 ± 0.5	236.3 ± 17.6	1.21
L2	87.9 ± 2.2	236.7 ± 18.4	1.21
H0	85.5 ± 1.5	245.4 ± 12.1	1.29
H1	89.3 ± 0.9	260.8 ± 13.1	1.31
H2	86.9 ± 0.6	260.4 ± 5.4	1.34

Data are presented as means ± SD (n = 100 and 10 for initial and final weights); SGR, specific growth rate (%/day) = 100 × [(ln weight$_{final}$ – ln weight$_{initial}$) × days^{-1}]; L0, L1, and L2, low-lipid diets with 0, 1, and 2% CLA; H0, H1, and H2, high-lipid diets with 0, 1, and 2% CLA. There were no significant differences between dietary treatments as determined by two-way ANOVA.

TABLE 5
Significance (*P* Values) of Effects of Dietary CLA and Oil Content, and Their Interaction as Determined by Two-Way ANOVA.

	P Value		
	CLA	Oil	Interaction
Liver			
HUFA synthesis activity	**<0.0001**	**<0.0001**	**<0.0001**
Δ6 Desaturase expression	**0.0009**	**<0.0001**	**0.0156**
Δ5 Desaturase expression	**0.0031**	**0.0009**	0.5247
β-Oxidation activity	0.2757	**0.0136**	0.5943
CPT-I activity	0.8506	0.5573	0.8676
CPT-I expression	**0.0027**	**0.0010**	**0.0483**
PPARα expression	**0.0001**	**0.0347**	0.1144
PPARβ expression	**0.0004**	**0.0445**	0.1649
PPARγ expression	**0.0449**	0.2061	0.2370
Red muscle			
β-Oxidation activity	0.8133	0.3457	0.5257
CPT-I activity	**0.0355**	0.1511	0.4857
CPT-I expression	**0.0022**	0.7695	0.3469
PPARα expression	0.2603	**0.0172**	**0.0034**
PPARβ expression	0.1182	**<0.0001**	**0.0460**
White muscle			
β-Oxidation activity	0.0795	0.1473	0.8623
CPT-I activity	**0.0075**	**0.0001**	0.1409
CPT-I expression	**0.0077**	0.7394	0.2716
PPARα expression	**0.0292**	0.0518	0.0836
PPARβ expression	**0.0032**	**<0.0006**	0.0822

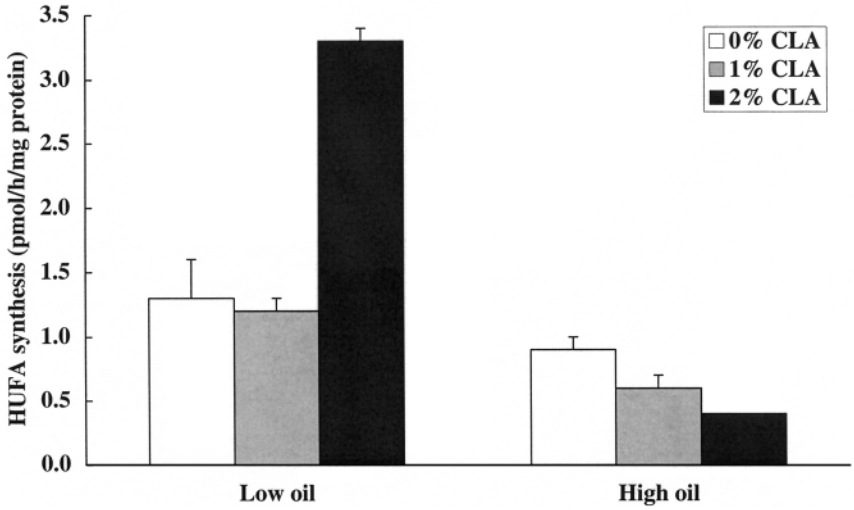

FIG. 1. Effects of dietary oil content and CLA on HUFA synthesis in the liver microsomes. Specific activities are presented as means ± SD (n = 3) and were determined as described in the text.

muscle was also significantly increased by high dietary oil. In contrast, hepatic CPT-I activity was unaffected by dietary oil content, but there was a trend for it to be decreased by dietary CLA in the low-oil group (Table 5, Fig. 3).

Tissue enzyme expression. The expression of FA Δ6 and Δ5 desaturases in liver was significantly affected by both dietary CLA and oil content (Table 5). Specifically, expression of both desaturases was increased by dietary CLA and reduced by high dietary oil content (Fig. 4). CPT-I expression in all three tissues, red and white muscle and liver, was significantly increased by dietary CLA (Table 5, Fig. 5). However, CPT-I expression was not affected by dietary oil content in either muscle tissue, although it was increased in liver tissue, at least in fish also being fed CLA (Table 5, Fig. 5).

Tissue PPAR expression. The expression of all PPAR subtypes was significantly affected by both dietary CLA and oil content (Table 5). Dietary CLA increased PPARα expression in liver and, to a lesser extent, white muscle (Fig. 6). In red muscle, there was a strong interaction between dietary CLA and oil content such that CLA increased expression at low oil content, but the opposite was true at high dietary oil content. High oil content may have induced increased expression of PPARα in red muscle, although the interaction also made this difficult to interpret. PPARβ expression in the muscle tissues was similar to that for PPARα in these tissues. Thus the expression of PPARβ was increased by dietary CLA in white muscle, whereas there was a significant interaction between CLA and dietary oil in red muscle, which made the effects of CLA difficult to interpret (Table 5). However, PPARβ expression was increased by high dietary oil, but dietary CLA decreased PPARβ expression in red muscle in fish fed high dietary oil (Fig. 7). The expression of PPARβ in liver was greatest in fish fed 1% CLA (Table 5, Fig. 7). Expression of PPARγ in liver was significantly increased by dietary CLA, but dietary oil content had no significant effect (Table 5, Fig. 8). PPARγ expression in muscle tissues was too low to be reliably determined.

DISCUSSION

CLA has the capacity to exert both agonistic and antagonistic effects on a wide variety of lipid metabolic factors. These effects depend on the pattern of lipid metabolism, which itself varies with species, tissue type, and age, and dietary factors, including lipid content, CLA isomer composition, and duration of supplementation. Thus, it is difficult to define a specific biochemical mechanism of action. However, the view that CLA can affect lipid accumulation both by decreasing *de novo* FA synthesis and by increasing oxidation is well established, at least in mammals. For example, CLA inhibits FA synthetase activity in rat liver (21), and suppresses TAG accumulation and increases FA oxidation in 3T3-L1 adipocytes (22). It is hypothesized that CLA exerts such effects on lipid metabolism at the transcriptional level by altering gene expression of key regulatory proteins and enzymes mediated, in part, by PPAR. However, it was unclear whether CLA have the ability to alter lipid metabolism in fish in a manner similar to that of mammalian models (23).

In the present study, salmon fed CLA exhibited increased expression of Δ5 and Δ6 FA desaturases in liver; this was particularly evident in the low-oil diet, which displayed an approximately twofold increase in expression of Δ5 and Δ6 at 2% inclusion of CLA. This was at least partly reflected in HUFA synthesis activity, which increased in fish fed 2% CLA at the low dietary oil content. Analogously, Δ5 and Δ6 FA desaturase expression was increased in mice fed CLA (24), whereas the amount of HUFA, EPA, and DHA increased in hybrid striped bass *Morone chrysops, saxatilis* in response to dietary CLA (25). One explanation may be that transcription factors equivalent to mammalian PPARα and SREBP-1c are directly involved in regulating Δ6 and Δ5 desaturases in fish via a feedback mechanism. A similar mechanism for the regulation of desaturase gene expression has been proposed in mammals, whereby Δ6 desaturase gene expression is partly

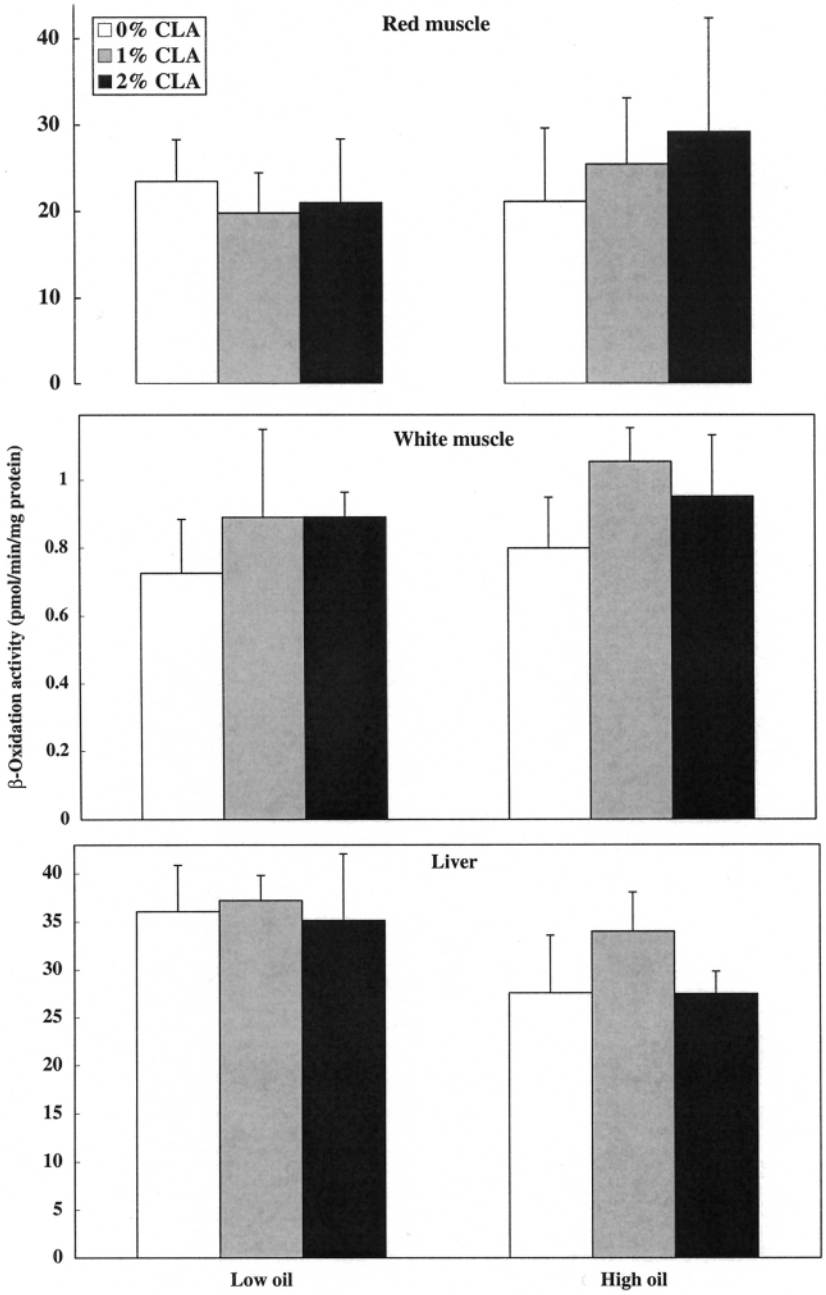

FIG. 2. Effects of dietary oil content and CLA on total β-oxidation activities in tissue homogenates of red and white muscle and liver. Specific activities are presented as means ± SD (n = 3) and were determined as described in the text.

regulated by PPARα (26). It was shown that ligand activation of PPARα via peroxisome proliferators may result in upregulation of Δ5 and Δ6 desaturase, and that high PUFA feeding downregulated these genes in a SREBP-1–dependent mechanism (11). In salmon liver, CLA feeding was associated with increases in Δ5 and Δ6 desaturase expression and HUFA synthesis in low-oil treatments, which might be indicative of a role for ligand activation of PPARα by CLA. In contrast, in high-oil diets, Δ5 and Δ6 expression and HUFA synthesis were repressed, even in the presence of similar amounts of CLA, possibly indicating a role for SREBP-1 proteins. Interestingly, CLA feeding also increased the levels of PPARα, providing some further support for this mechanism of regulation in salmon liver.

Previously, however, we reported that dietary CLA had no significant effect on either liver or flesh (muscle) FA compositions in salmon; thus it appears that the increased FA desaturase expression and HUFA synthesis activity does not have a major physiological consequence in terms of gross FA compositions (16). That alterations in desaturase gene expression

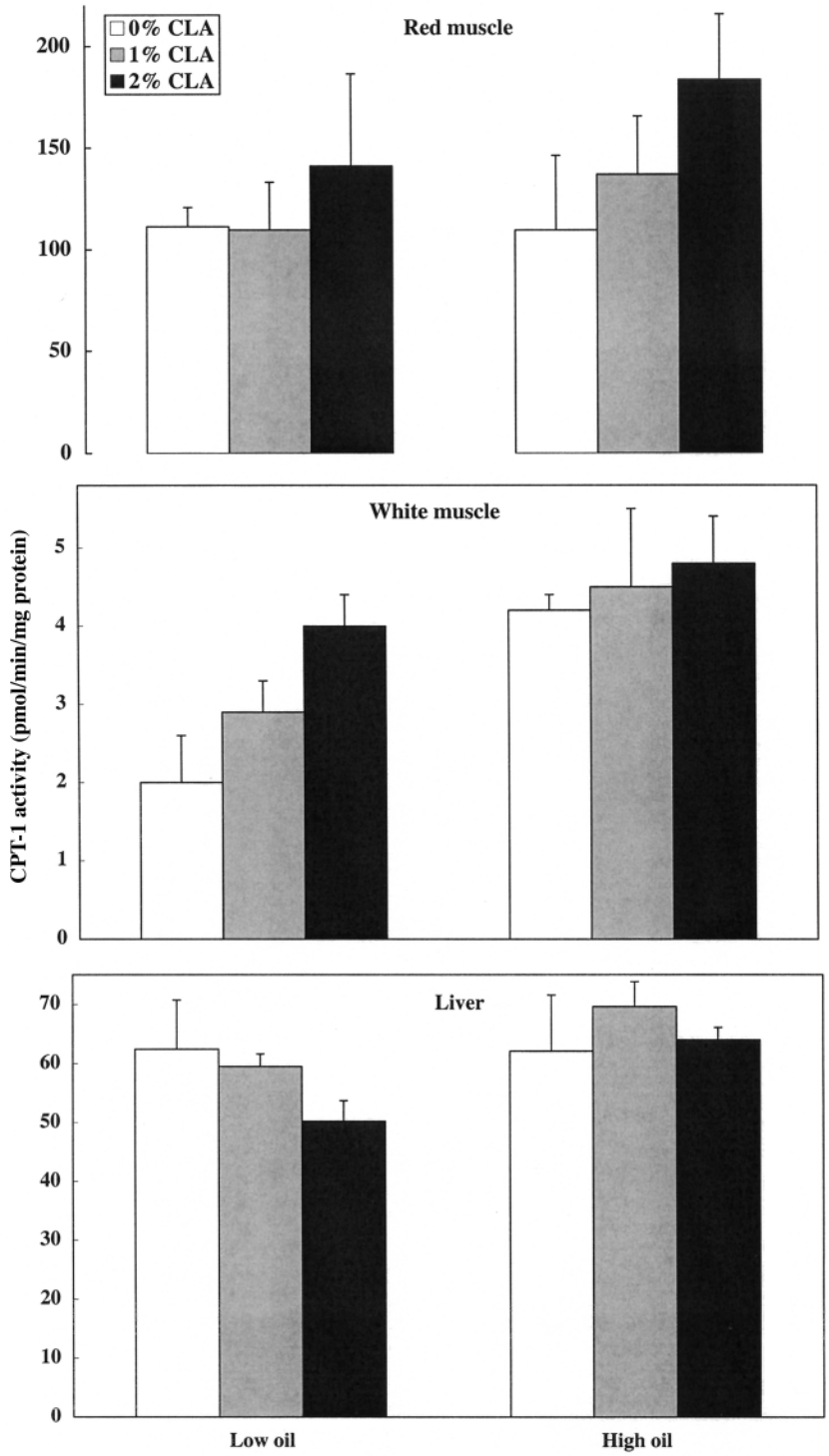

FIG. 3. Effects of dietary oil content and CLA on CPT-I activities in tissue homogenates of red and white muscle and liver. Specific activities are presented as means ± SD (n = 3) and were determined as described in the text.

and HUFA synthesis activity in liver can have relatively little effect on tissue FA compositions has been demonstrated previously in several trials in which salmon have been fed vegetable oils (see 27). The data from the present trial also show a correlation between desaturase expression and HUFA synthesis activity, and dietary oil content, with diets containing high oil conferring significantly lower levels of desaturase expression and HUFA synthesis activity compared to low-oil diets. In addition to effects on HUFA synthesis, it is well established that there is a reduction in FA synthesis (lipogene-

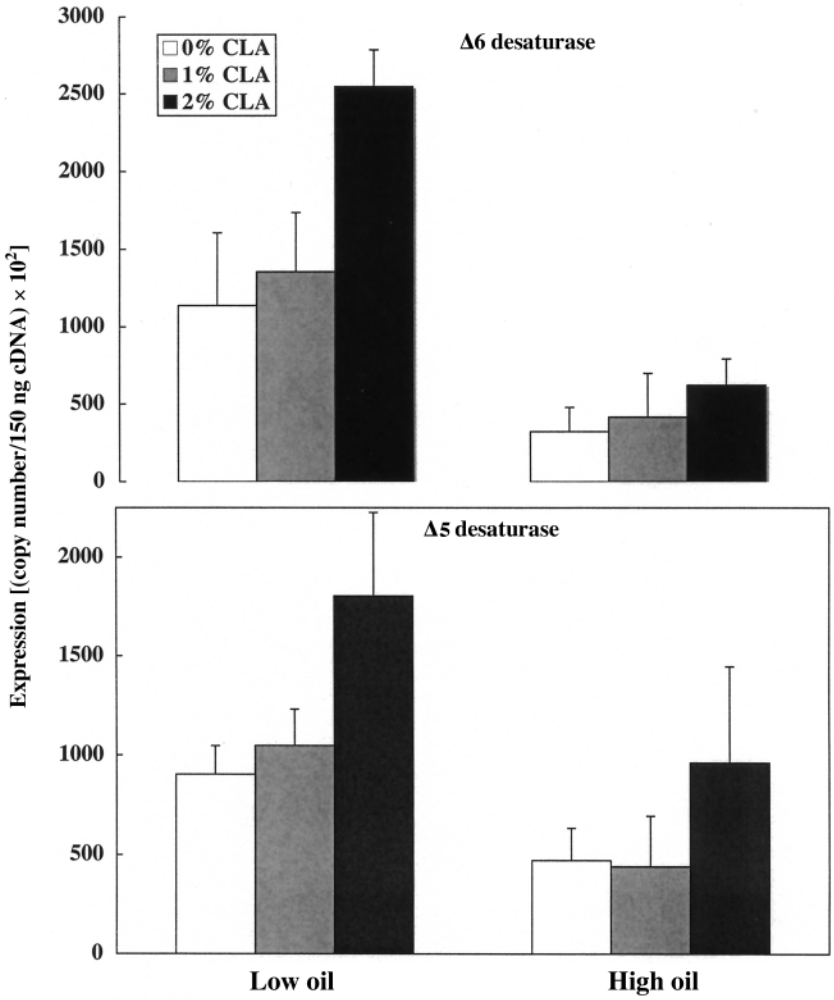

FIG. 4. Effects of dietary oil content and CLA on the expression of Δ6 and Δ5 FA desaturase genes in liver. Genes were determined by rtqPCR and normalized relative to total RNA, determined by fluorescent assay. Results are presented as means ± SD (n = 3).

sis) as a result of high dietary oil levels, as demonstrated in Atlantic salmon and other fish species (28).

β-Oxidation is the principal means of FA catabolism in vertebrates. The basic mechanism of the pathway, and possibly regulation, appear to be highly conserved in mammals and fish (29). Recently, it has been suggested that CLA can increase mitochondrial β-oxidation in a variety of tissues in rats via an increase in CPT-I (30). Moreover, evidence of increased FA oxidation after CLA supplementation is presented in similar studies, which indicated that CLA increases gene expression and activity of CPT-I in mouse liver (31). However, in the present trial, β-oxidation capacity was not significantly affected in red or white muscle tissue of fish fed dietary CLA, despite mitochondrial β-oxidation predominating over peroxisomal β-oxidation in salmon red and white muscle (32). In contrast, CLA significantly increased both CPT-I expression and activity in these tissues. The reasons for the lack of association of CPT-I with β-oxidation are unclear. Increased CPT-I activities in liver and red muscle of hamsters fed CLA was not correlated with increased FA oxidation (33). The authors speculated that this may have been due to increased sensitivity of CPT-I to malonyl-CoA in animals fed CLA (33). As in mammals, malonyl-CoA is an inhibitor of CPT-I in Atlantic salmon, but it is not known if CLA can affect its potency (34). There are few data in fish species to compare the present data with. However, dietary lipid level did not alter β-oxidation activity in the muscle of haddock *Melanogrammus aeglefinus* L. fed graded levels of oil (29). In the present study, dietary oil content had no major effect on β-oxidation activity or CPT-I mRNA levels in either red or white muscle, or CPT-I activity in red muscle, although CPT-I activity was increased by dietary oil content in white muscle.

Previous studies in rodents showed that CPT-I mRNA level was elevated in liver in response to dietary CLA (31). In the present study and similar to results in muscle, CPT-I gene expression was found to be upregulated in salmon liver in response to dietary CLA, although this was not accompanied by

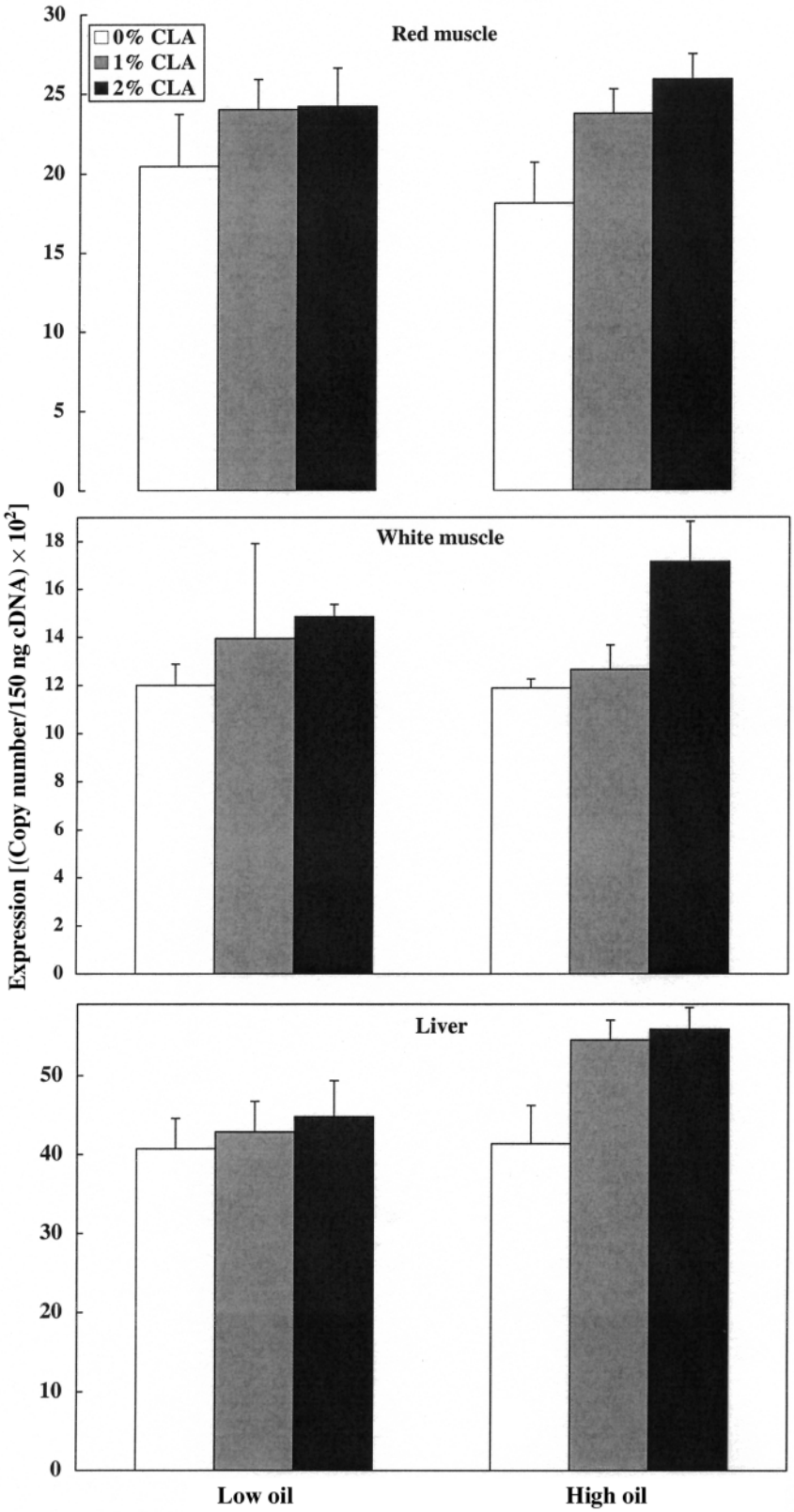

FIG. 5. Effects of dietary oil content and CLA on the expression of CPT-I in the liver, red muscle, and white muscle. Genes were determined by rtqPCR and normalized relative to total RNA, determined by fluorescent assay. Results are presented as means ± SD (n = 3).

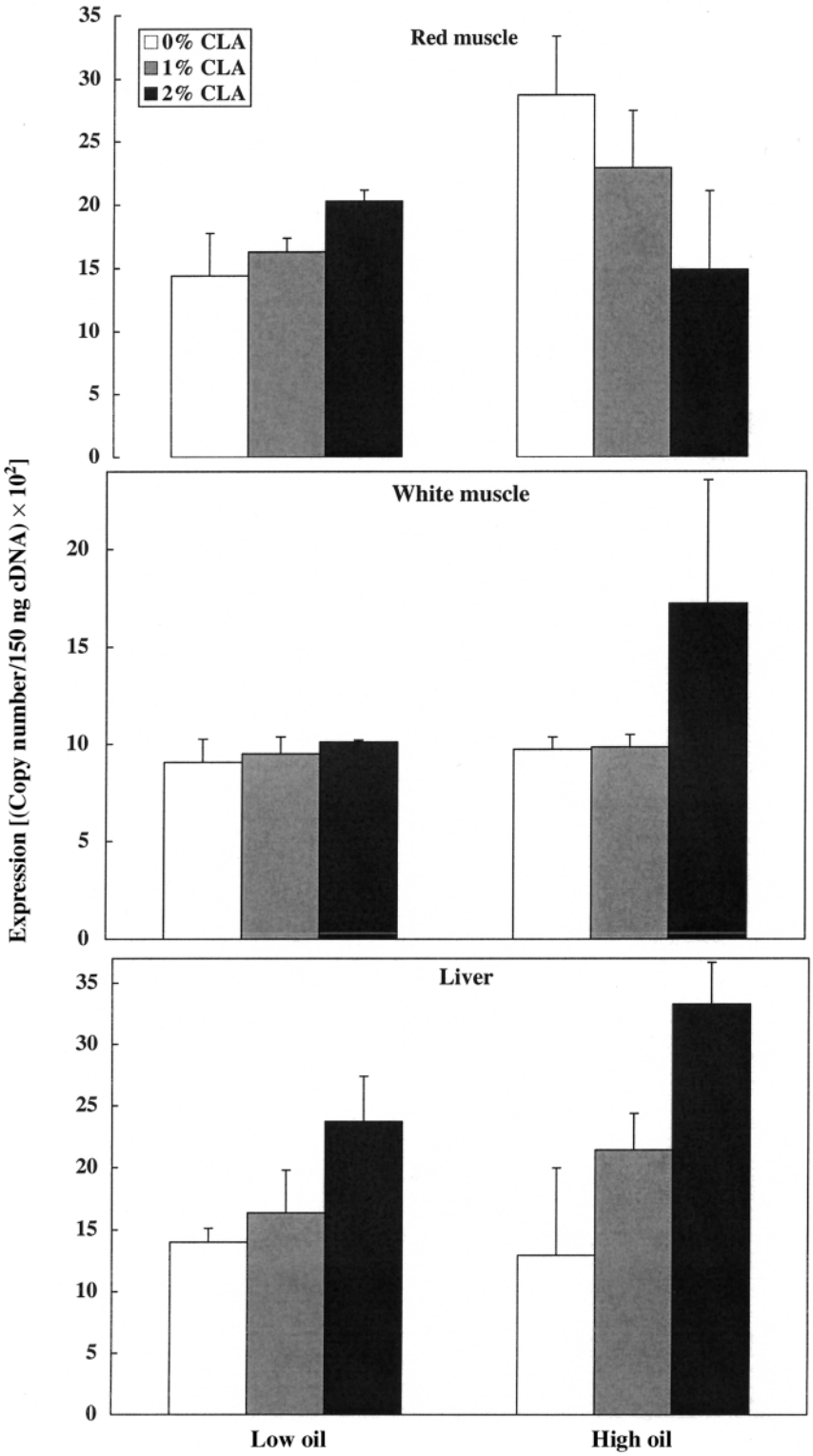

FIG. 6. Effects of dietary oil content and CLA on the expression of PPARα in the liver, red muscle, and white muscle. Genes were determined by rtqPCR and normalized relative to total RNA, determined by fluorescent assay. Results are presented as means ± SD (n = 3).

increased CPT-I or β-oxidation activities. Furthermore, CPT-I expression was higher in salmon liver in fish fed the high-oil diet when fed in combination with CLA. Consistent with this, it appeared that in fish fed CLA, CPT-I activity was lower in fish fed the low-oil diet compared with fish fed the high-oil diet. However, these effects on CPT-I expression and activity

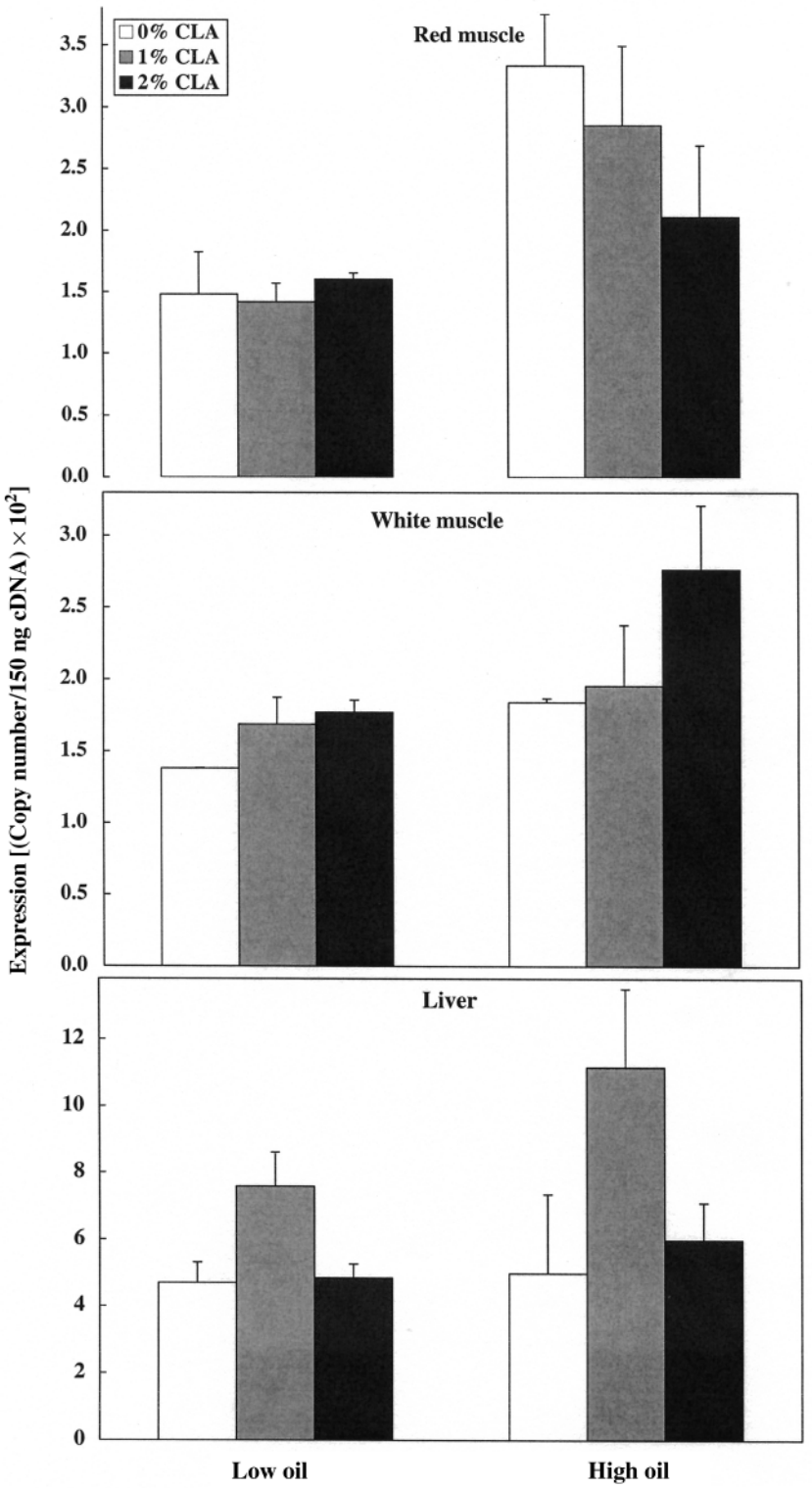

FIG. 7. Effects of dietary oil content and CLA on the expression of PPARβ in the liver, red muscle, and white muscle. Genes were determined by rtqPCR and normalized relative to total RNA, determined by fluorescent assay. Results are presented as means ± SD (n = 3).

in liver were not reflected in β-oxidation activity, which surprisingly was reduced in fish fed the high-oil diet. These results seem contradictory considering that the *in vivo* rate of FA oxidation should be associated with *in vitro* CPT-I activity (35). Nevertheless, studies determining changes in energy metabolism in hamsters fed CLA also found disparities between the

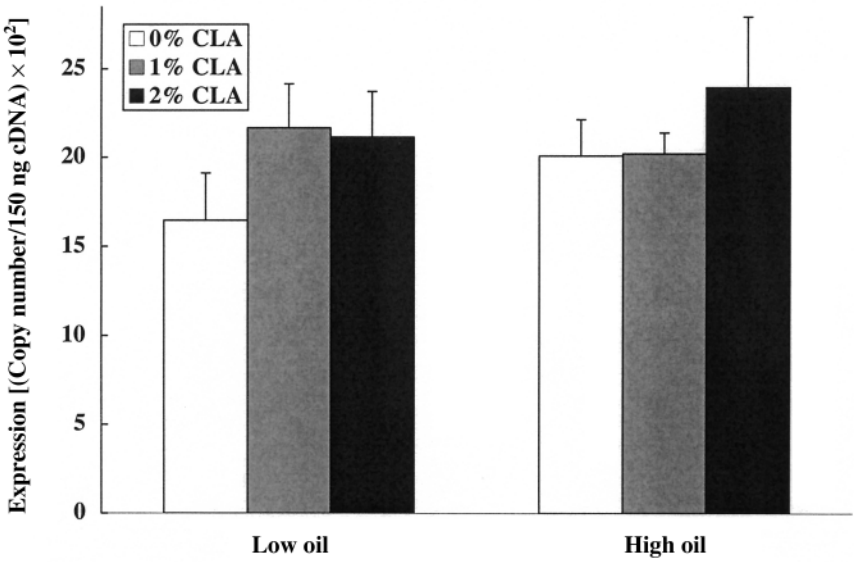

FIG.8. Effects of dietary oil content and CLA on the expression of PPARγ in liver. Gene expression was determined by rtqPCR and normalized relative to total RNA, determined by fluorescent assay. Results are presented as means ± SD (n = 3).

activity of CPT-I and lipid oxidation in the liver (33). Pertinent to this lack of correlation between CPT-I expression and activity and β-oxidation activity in liver is the fact that, with Atlantic salmon of similar age and size, catabolism of FA in liver was reported to be principally due to peroxisomal, rather than mitochondrial, β-oxidation (32). Consequently, CPT-I may not have a prominent role as a regulatory element of FA oxidation in salmon smolt liver, considering it is primarily involved in mitochondrial metabolism (36). This may be relevant in the present study, as it clearly provides a mechanism whereby changes in CPT-I expression and/or activity can be unrelated to β-oxidation activity in liver. This remains the case even considering that, in the present study, only total β-oxidation, combining mitochondrial and peroxisomal β-oxidation, was determined. Clearly, however, it also suggests that peroxisomal β-oxidation in salmon liver was unaffected by dietary CLA, and was actually decreased by high dietary oil. In comparison, graded levels of dietary lipid showed no significant differences in β-oxidation activity in liver of haddock (29).

There is consolidating evidence that lipid homeostasis is, at least in part, modulated through the PPAR transcription factors in mammals. Recent studies suggest that CLA could mediate this activation by acting as high-affinity ligands for a number of PPAR isotypes, particularly PPARα (7). PPARα is intimately involved in the regulation of genes involved in mitochondrial FA oxidative processes in mammals (37), whereas PPARγ is primarily involved in lipid deposition via preadipocyte differentiation and lipogenesis (38). Very recent work has elucidated that marine fish also share homologous gene sequences, with similar phylogenetic characteristics to the mammalian PPAR counterparts, possibly also suggesting similar molecular roles (39,40). However, Atlantic salmon may possibly contain up to five PPAR genes, as opposed to three in mammals, and therefore the precise role of piscine PPARs have not been conclusively defined (40).

The effects of CLA on mammalian PPAR gene expression are ambiguous and seem to be dependent partly on species, partly on tissue type, and partly on CLA isomer. The majority of studies describe a decrease in PPARγ mRNA in isolated adipocytes, or in adipose tissue from mice treated with *trans*-10,*cis*-12 CLA or a 1:1 mixture of the two predominant CLA isomers (41,42). Conversely, CLA has also been reported to increase expression of PPARγ in liver of mice, and in white adipose tissue of rats (43,44). *Trans*-10,*cis*-12 CLA downregulated PPARα in mice (45); however, there was no change in PPARα expression in liver of hamsters fed diets containing *trans*-10,*cis*-12 or *cis*-9,*trans*-11 CLA (46). In the present study, there was a trend for PPARα expression to be increased by dietary CLA in liver, red muscle, and white muscle of fish fed the low-oil diets. In addition, upregulation of PPARα in response to dietary CLA was also observed in white muscle and liver in fish fed the high-oil diets. As previously mentioned, activation of PPARα (via peroxisome proliferators) can induce β-oxidation through upregulation of key enzymes such as CPT-I in mammals (37). The data in the present trial suggest that PPARα may also be implicated in the action of CLA. In general, CLA increased the levels of both CPT-I mRNA and PPARα, and in some cases PPARβ mRNA. The exceptions to this were PPARα and PPARβ expression in red muscle from fish fed high oil, which tended to decrease with dietary CLA inclusion, and at high CLA inclusion liver PPARβ was decreased. It has been shown in PPARα-knockout mice that the effects of CLA on body fat distribution and mitochondrial lipid catabolism genes are not mediated by PPARα, but that the peroxisomal β-oxidation gene acyl CoA oxidase is regulated by a PPARα-dependent mechanism (47).

Acyl CoA oxidase shows high activity in salmon liver, but much lower activity in red muscle and very low activity in white muscle (34). Thus, it is possible that effects on mitochondrial lipid catabolic genes such as CPT-I could be mediated through PPARβ subtypes. However, PPARα expression exceeds that of PPARβ by an order of magnitude in muscle tissues, suggesting that PPARα would have the predominant regulatory role. Of course, it is not the absolute amount of PPAR that is important for target gene activation, but rather the binding of activating ligand.

Of the tissues investigated in salmon, PPARγ expression was only detected in liver. However, in agreement with work carried out in mice (43), PPARγ mRNA levels increased in liver of salmon fed CLA, and CLA have been reported to cause fatty liver in mice (43). However, PPARγ in fish does not share the same ligand activation profile as PPARγ in mammals (40). Fish PPARγ does not respond to FA and has specific amino acid differences compared to the mammalian form, which explains lack of activation, suggesting that it is unlikely, despite increases observed in liver, that CLA would mediate its effects through this transcription factor. Indeed, neither liver size nor lipid content was increased in salmon fed CLA (16).

In summary, gene expression and activity of various lipid metabolic factors were altered in response to graded levels of CLA and/or dietary oil content in Atlantic salmon smolts. Specifically, some association was observed between dietary CLA, liver HUFA synthesis and desaturase gene expression, and liver PPARα expression, although this varied with dietary oil content. In addition, some association between dietary CLA, CPT-I expression and activity, and PPARα expression was observed in muscle tissues. However, the magnitude of the changes in FA metabolism observed was not sufficient to bring about major changes in the whole body lipid and FA composition of the fish (16). In conclusion, this study has presented evidence that dietary CLA have effects on FA metabolism in Atlantic salmon, but that there is little evidence of a direct mechanism involving PPAR transcription factors. However, considering the importance of dietary lipid in aquaculture, further work is required to assess the potential of CLA as a dietary supplement, and the role of PPAR in the regulation of lipid metabolism in fish.

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Conjugated Linoleic Acid and Chromium Lower Body Weight and Visceral Fat Mass in High-Fat-Diet–Fed Mice

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ABSTRACT: More than half of the U.S. population has a body mass index of 25 kg/m^2 or more, which classifies them as overweight or obese. Obesity is often associated with comorbidities such as diabetes, cardiovascular diseases, and cancer. CLA and chromium have emerged as major dietary supplements that reduce body weight and fat mass, and increase basal metabolic rate in animal models. However, studies show that CLA induces insulin resistance in mice and in humans, whereas Cr improves insulin sensitivity. Hence, we designed the present study to examine the combined effect of CLA and Cr on body composition and insulin sensitivity in a Balb/c mice (n = 10/group) model of high-fat-diet–induced obesity. CLA alone lowered body weight, total body fat mass, and visceral fat mass, the last of which decreased further with the combination of CLA and Cr. This effect was accompanied by decreased serum leptin levels in CLA-fed and CLA + Cr–fed mice, and by higher energy expenditure (EE) and oxygen consumption (OC) in CLA + Cr–fed mice. Serum levels of glucose, insulin, the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), as well as insulin resistance index (IRI), decreased with CLA, whereas CLA and Cr in combination had significant effects on insulin and IL-6 concentrations and IRI. In summary, CLA + Cr decreased body weight and fat mass in high-fat-diet–fed mice, which may be associated with decreased leptin levels and higher EE and OC.

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Obesity is one of the major avoidable risk factors for mortality and morbidity, and increases the risk for type 2 diabetes, cancer, diseases associated with the cardiovascular system, and osteoarthritis in weight-bearing joints (1), leading to an enormous increase in health care costs. Decrease in energy expenditure (EE) associated with lack of physical activity has been identified as one of the major reasons for the spread of this epidemic (2–5). Obesity is usually accompanied by abnormalities in leptin and insulin secretion and their action, together with defects in lipid and carbohydrate metabolism (6,7).

CLA refers to a group of polyunsaturated FA that are positional and stereoisomers of octadecadienoic acid (linoleic acid, LA, 18:2n-6, double bonds at position 9 and 12). They are found naturally in ruminant food products such as beef, lamb, and dairy products containing approximately 80% *cis*-9, *trans*-11 (c9t11) isomer and 10% *trans*-10, *cis*-12 (t10c12) isomer (8,9). CLA is available commercially as a mixture of c9t11 and t10c12 isomers present in equal amounts (~35%), and has significant antiadipogenic, anticarcinogenic, antiatherosclerotic, antiinflammatory, antidiabetic, and antihypertensive properties in animal models (10–18). Some recent studies also indicate that CLA reduces body fat mass (BFM) in humans (19,20). However, evidence derived from studies in mice and humans suggest that intake of CLA or t10c12 isomer alone may induce hyperglycemia, hyperinsulinemia, and insulin resistance (IR) (16,21–25). In a recent study in human mature adipocytes, t10c12 isomer activated nuclear factor-kappaB (NF-κB) and ERK1/2-dependent tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production, which suppressed peroxisome proliferator-activated receptor-γ (PPAR-γ), leading to impaired glucose uptake. This provided supportive evidence that the t10c12 isomer induces IR (26).

Chromium is an important trace element essential for normal fat, carbohydrate, and protein metabolism (27). Chromium intake increases lean mass and decreases body weight and fat mass in animal studies and in humans (27,28). Moreover, it improves serum lipid levels and glucose tolerance in rodents and in humans, and increases or potentiates insulin's effects on glucose uptake and utilization (29–33) and decreases IR (34,35).

It is well established that rodents on a high-fat diet develop obesity and IR, similar to the effect of Western—style diet in humans (36–38). Because CLA and Cr have beneficial effects on body composition, we hypothesized that intake of a low dose of CLA and Cr, in combination, would decrease fat mass in a mouse model of high-fat-diet–induced obesity. We also hypothesized that CLA could induce hyperinsulinemia and hyperglycemia in this mouse model, which may be amelio-

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Abbreviations: AKP, alkaline phosphatase; BFM, total body fat mass; BLM, total body lean mass; CO, corn oil; c9t11, *cis*-9, *trans*-11; DEXA, dual energy x-ray absorptiometry; EE, energy expenditure; IL, interleukin; IR, insulin resistance; IRI, insulin resistance index; IRK, insulin receptor kinase; IRTP, insulin receptor tyrosine phosphatase; IS, insulin sensitivity; LA, linoleic acid; NF-κB, nuclear factor-kappaB; OC, oxygen consumption; PPAR-γ, peroxisome proliferator-activated receptor-γ; SFO, safflower oil; t10C12, *trans*-10, *cis*-12; TNF, tumor necrosis factor; UCP2, uncoupling protein 2; VFM, visceral fat mass.

rated by Cr intake. To establish this hypothesis, we conducted the present study in Balb/c mice on a high-fat diet (20% corn oil [CO], ~35% kcal) that induced obesity. Balb/c mice significantly gain body weight and fat mass in response to high-fat diet (39). We performed a longitudinal study to measure the interaction of CLA and Cr in their effects on body composition utilizing dual-energy x-ray absorptiometry (DEXA) technology. We measured serum levels of glucose, insulin, leptin, adiponectin, the proinflammatory cytokines, IL-6, and TNF-α, and insulin resistance index (IRI). Because CLA increases EE (40,41), we also measured parameters of metabolic rate to determine whether any CLA + Cr–mediated decrease in fat mass is associated with higher EE and oxygen consumption (OC).

EXPERIMENTAL PROCEDURES

Animals and experimental diets. Six-week-old male Balb/c mice were obtained from Harlan (Indianapolis, IN). Weight—matched mice were housed in a laboratory animal care facility in cages (5 mice/cage) and fed standard lab chow diet. At 8 wk of age, animals were divided into different groups and provided with semipurified AIN-93M diets containing 0.5% safflower oil (SFO) or 0.5% CLA (Clarinol powder, Loders Croklaan, Channahon, IL). Each group was further divided into Cr and non-Cr groups of 10 mice each, thus resulting in four treatment groups: SFO, SFO + Cr, CLA, and CLA + Cr . The diets contained 20% CO by weight. The composition of the semipurified diets is provided in Table 1. The composition of the oil extracted from the Clarinol powder was: c9t11, 36.9%; t10c12, 37.4%; total trans, 2.7%. The total CLA content of the oil was 80.9%, with the main isomers accounting for 74.4%. Like CO, SFO contains 18:2n-6 (LA) as the major FA component. Cr was provided as chromium tricarnosinate at 22.77 g/kg diet (3.4% chromium and 89.3% carnosine, FutureCeutical Carnochrome, VDF FutureCeuticals, Momence, IL). Fresh diet was prepared weekly, stored in aliquots at –20ºC, and provided daily *ad libitum*. Body weight was monitored biweekly. Animals were maintained on a 12—hr light/12—hr dark cycle in an ambient temperature of 22–25ºC at 45% humidity. NIH guidelines (42) were strictly followed; and the Institutional Laboratory Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio approved all studies.

TABLE 1
Composition of Experimental Diets

Ingredients (g/kg)	SFO	CLA	SFO + Cr	CLA + Cr
Casein	248	248	248	248
AIN-93 mineral mix	64	64	64	64
AIN-93 vitamin mix	17	17	17	17
Cellulose	55	55	55	55
Anhydrous dextrose	405.5	403.74	382.73	380.97
Choline bitartrate	2.5	2.5	2.5	2.5
DL-Methionine	3.0	3.0	3.0	3.0
Clarinol powder[a]	0	6.76	0	6.76
Safflower oil	5.0	0	5.0	0
Chromium cerelose[b]	0	0	22.77	22.77
Corn oil	200	200	200	200
Total	1000	1000	1000	1000

[a]Contains 74% CLA.
[b]CarnoChrome (chromium tricarnosinate), 3.14% (wt/wt); chromium mixed 3 mg/10 g in cerelose.

Collection of tissues and blood serum. Blood was collected after overnight fasting under anesthesia by retro-orbital bleeding 3 d before the end of the experiment, and serum was obtained by centrifugation at 3000 rpm for 15 min at 4°C for the determination of glucose and insulin. At the termination of the study, mice were sacrificed by cervical dislocation, and serum was collected for all the other assays.

Measurement of fat mass and lean mass. BFM and total body lean mass (BLM) were measured by DEXA using a Lunar PIXImus mouse bone densitometer (GE**,** Madison, WI), and data were analyzed with PIXImus software (43—45). Before scanning was performed, mice were anesthetized with an intramuscular injection of 0.1 mL/100 g body weight of mouse cocktail containing ketamine/S.A. rompun/NaCl (3:2:5, by vol). BFM and BLM were obtained for the entire body, excluding the head. Visceral fat mass (VFM) was determined manually using DEXA. Scanning was performed first at baseline and again at the end of 14 wk of the study diet.

Serum metabolites. Insulin was analyzed using a rat/mouse insulin ELISA kit from Linco Research (St Charles, MO). Glucose was analyzed spectrophotometrically using Sigma Diagnostics glucose (Trinder) reagent (Sigma Diagnostics, St Louis, MO). Adiponectin was assayed using mouse adiponectin Quantikine immunoassay kit from R&D Systems (Minneapolis, MN). Leptin was assayed using an active murine leptin kit from Diagnostic Systems Laboratories (Webster, TX).

IRI. IRI was calculated by the following formula: [fasting serum insulin (ng/mL) × fasting serum glucose (mM)]/22.5. A high IRI denotes low insulin sensitivity (IS) (46).

Serum proinflammatory cytokines. TNF-α and IL-6 were measured by using BD OptEIA™ ELISA kits from BD Biosciences Pharmingen (San Diego, CA) (43). Sensitivity of the assays was approximately 1 pg/mL. In brief, each well of flat-bottom 96-well microtiter plates was coated with 100 µl of purified anti-TNF-α and anti-IL-6 antibodies (4 µg/mL in binding solution) overnight at 4°C. The plates were rinsed four times with washing buffer, and diluted serum was added, followed by incubation for 2 h at room temperature. The plates were washed four times with washing buffer, followed by the addition of biotinylated anticytokine antibodies. The plates were incubated in room temperature for 1 h and then washed four times with washing buffer. Streptavidin-alkaline phosphatase conjugate was added, and the plates were incubated for 30 min at room temperature. The plates were again washed four times with washing buffer, and the chromogen substrate was added. The plates were then incubated at room temperature to achieve the desired maximum absorbance and were read at 410 nM in an ELISA reader (Dynex Technologies, Worthington, West Sussex, United Kingdom).

Metabolic rate. Metabolic rate was measured indirectly by

drawing air through sealed rodent cages and monitoring the partial pressures of oxygen and carbon dioxide of the air entering and leaving the cage, as described in several earlier studies (47). In brief, gas composition was measured using a zirconia cell O_2 detector and an infrared CO_2 analyzer (Applied Electrochemistry models S-3A and CD-3A, respectively; Ametek, Paoli, PA), with flow regulated by a mass flow controller (Linde model FM4570; Ontario, Canada). Gas pressures, airflow, cage temperature, ambient air temperature, pressure, and humidity were digitized, recorded every 10 s, and averaged on an hourly basis. Each mouse was monitored between 0800 and 1200 h for 3 h. Mice were acclimated to the flowthrough system for 24 h before measurement of metabolic rate. During the time of measurement, air was also sampled from an empty cage situated adjacent to the experimental cage, as a control. The rate of O_2 consumption was calculated using the equations of Consolazio et al. (48). The metabolic rate was then calculated in kJ/min using these values and an energy equivalent of 1 L of oxygen per 4.76 kJ. This assumes that the fuel combusted *in vivo* is the same as that of the food ingested; only small errors are introduced if the fuel mix metabolized at a given time deviates from this value.

Statistical analysis. Results are expressed as means ± SEM. Data were analyzed statistically by two-way ANOVA or repeated-measures ANOVA using Graphpad Prism 4 software. The Newman-Keuls multiple comparison test was used to test the differences among groups. Differences were considered significant at $P < 0.05$.

RESULTS

Body composition and liver weight. Body weight initially increased in all the dietary groups until 6 wk of age. Thereafter, body weight increased maximally in SFO-fed mice, whereas body weight decreased in CLA + Cr–fed mice (Fig. 1). Body weight was significantly lowered in CLA-fed mice, compared with SFO-fed mice ($P < 0.001$). Cr decreased body weight in both SFO-fed and CLA-fed mice but more significantly in CLA + Cr–fed mice. Body weight in SFO + Cr–fed mice was comparable to CLA-fed mice. CLA-fed mice showed lower fat mass that decreased further in CLA + Cr–fed mice ($P < 0.05$) compared with SFO-fed mice. VFM is the sum of the weights of the mesenteric, epididymal, and retroperitoneal fat depots. VFM was significantly lowered in CLA-fed mice compared with SFO-fed mice, and decreased further in CLA + Cr–fed mice ($P < 0.05$). There was no difference in liver weights between the different dietary groups (Table 2). Lean mass was higher in CLA-fed mice compared with SFO + Cr–fed and CLA + Cr–fed mice but not SFO-fed mice (Table 2).

Serum metabolites. Leptin was lowered in CLA-fed and CLA + Cr–fed mice compared with SFO-fed and SFO + Cr–fed mice but there was no effect of Cr supplementation ($P < 0.001$). Serum adiponectin levels were unaltered by CLA or Cr. Glucose concentrations was lowered in CLA-fed mice

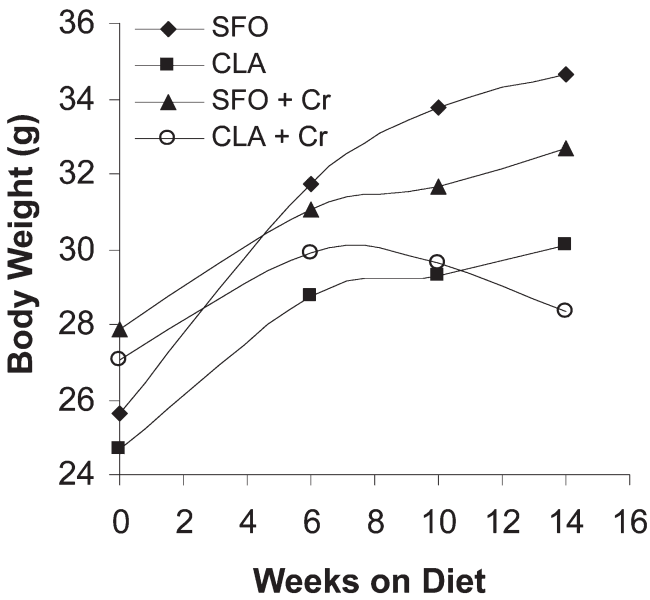

FIG. 1. Body weight curves for safflower oil (SFO) control, conjugated linoleic acid (CLA)–treated, SFO and chromium (Cr) (SFO + Cr)–treated, and CLA and Cr (CLA + Cr)–treated mice ($n = 10$/group). Day 0 was the first day mice were placed on different diets. Both CLA and Cr significantly suppressed body weight (CLA: $P < 0.0001$; Cr: $P < 0.05$).

compared with SFO-fed and SFO + Cr–fed mice ($P < 0.05$). However, glucose level in the CLA + Cr group was comparable to that in all the other dietary groups. Analysis by two-way ANOVA for main effects revealed that the only statistically significant effect of CLA was on glucose level. Insulin levels decreased in SFO + Cr–fed, CLA-fed, and CLA + Cr–fed mice compared with SFO-fed mice. Analyzing the data by two-way ANOVA for main effects showed that CLA and the combination of CLA and Cr had significant effects on insulin levels. Insulin sensitivity was estimated by several indices, including the homeostasis model assessment, l/insulin, and glucose/insulin. A higher IRI reflects low IS; IRI was lowered in all the other dietary groups compared with SFO-fed mice. However, the decrease was much higher in CLA-fed and CLA + Cr–fed mice. Analyzing the data by two-way ANOVA for main effects showed significant effects of CLA, Cr, and their interaction on IRI (Table 3).

Serum proinflammatory cytokines. Serum TNF-α levels were lowered in CLA-fed and CLA + Cr–fed mice compared with SFO-fed and SFO + Cr–fed mice ($P < 0.05$), but there was no additional effect of Cr. Two-way ANOVA for main effects revealed that the only statistically significant effect of CLA was on TNF-α, whereas interaction of CLA and Cr was close to being statistically significant. Serum IL-6 levels were decreased in SFO + Cr–fed, CLA-fed, and CLA + Cr–fed mice compared with SFO-fed mice. Cr lowered IL-6 in SFO-fed mice but there was no additional effect of Cr in CLA-fed mice. Analyzing the data by two-way ANOVA for main effects found significant effects of CLA and interaction of CLA and Cr on IL-6 activity (Table 3).

OC and EE. OC and EE did not differ between SFO-fed

TABLE 2
Body Composition and Liver Weights in Male Balb/c Mice Fed Control (SFO) or CLA Diets With or Without Chromium (Cr) Supplementation for 14 wk[a]

					P Value[b]		
	SFO	CLA	SFO + Cr	CLA + Cr	CLA	Cr	CLA × Cr
Body weight, g							
Baseline	25.64 ± 0.80	24.67 ± 0.73	27.84 ± 0.87	27.08 ± 0.99			
Final	34.67 ± 1.20[a]	30.19 ± 0.58[bc]	32.66 ± 0.88[ac]	28.35 ± 0.59[b]	<0.0001	0.04	0.93
Increase in body weight	9.03 ± 0.93[a]	5.54 ± 0.46[b]	4.82 ± 0.77[b]	1.25 ± 0.49[c]			
Total body fat mass, g							
Baseline	3.06 ± 0.15[a]	2.47 ± 0.07[b]	3.57 ± 0.17[c]	3.69 ± 0.22[c]			
Final	8.10 ± 0.75[a]	3.74 ± 0.33[b]	5.91 ± 0.74[c]	3.13 ± 0.24[b]	<0.0001	0.03	0.20
Increase in fat mass	5.02 ± 0.65[a]	1.24 ± 0.33[b]	2.34 ± 0.85[b]	–0.45 ± 0.24[c]			
Total body lean mass, g							
Baseline	16.38 ± 0.19[a]	16.23 ± 0.22[a]	17.63 ± 0.32[b]	17.40 ± 0.33[b]			
Final	19.39 ± 0.55[ab]	20.25 ± 0.38[a]	20.48 ± 0.30[a]	18.71 ± 0.34[b]	0.28	0.60	<0.01
Increase in lean mass	3.01 ± 0.53[ab]	4.08 ± 0.31[b]	2.87 ± 0.32[a]	1.04 ± 0.42[c]			
Visceral fat mass, g							
Baseline	0.77 ± 0.07	0.50 ± 0.04	0.89 ± 0.06	1.00 ± 0.08			
Final	2.65 ± 0.16[a]	0.81 ± 0.17[b]	1.72 ± 0.16[c]	0.66 ± 0.18[b]	<0.0001	0.04	0.15
Increase in visceral fat mass, g	1.87 ± 0.13[a]	0.30 ± 0.05[b]	0.83 ± 0.06[c]	–0.32 ± 0.08[d]			
Liver weight, g	1.37 ± 0.08	1.44 ± 0.07	1.41 ± 0.04	1.47 ± 0.05	0.13	0.22	0.24

[a]Values are means ± SEM, n = 10. Means in a row with superscripts without a common letter differ by Newman–Keuls multiple comparison test, $P < 0.05$.
[b]From two-way ANOVA with repeated measures at 14 wk.

TABLE 3
Serum Parameters in Male Balb/c Mice Fed Control (SFO) or CLA Diets With or Without Chromium (Cr) Supplementation for 14 wk[a]

					P Value[b]		
	SFO	CLA	SFO + Cr	CLA + Cr	CLA	Cr	CLA × Cr
Glucose							
mg/dL	162.70 ± 6.15[a]	138.10 ± 4.55[b]	169.30 ± 5.66[a]	154.80 ± 6.58[ab]	<0.01	0.06	0.39
mM	9.02 ± 0.34[a]	7.66 ± 0.25[b]	9.39 ± 0.31[a]	8.59 ± 0.36[ab]	<0.01	0.06	0.39
Insulin (ng/mL)	20.10 ± 2.02[a]	7.60 ± 0.31[b]	11.64 ± 0.74[b]	11.04 ± 1.49[b]	<0.001	0.08	<0.001
Insulin Resistance Index[c] (ng/mL × mM)	7.96 ± 0.58[a]	2.59 ± 0.17[b]	5.11 ± 0.07[c]	3.66 ± 0.49[b]	<0.0001	0.04	<0.001
Leptin (ng/mL)	2.42 ± 0.06[a]	1.61 ± 0.07[b]	2.45 ± 0.10[a]	1.91 ± 0.16[b]	<0.0001	0.13	0.23
Adiponectin (μg/mL)	3.53 ± 0.32	3.88 ± 0.11	4.03 ± 0.14	3.90 ± 0.28	0.61	0.28	0.32
TNF-α (pg/mL)	252.8 ± 18.21[a]	108.5 ± 2.83[b]	199.23 ± 1.50[a]	131.0 ± 16.15[b]	<0.001	0.46	0.08
IL-6 (pg/mL)	106.00 ± 10.97[a]	64.50 ± 1.46[b]	80.63 ± 1.88[b]	71.00 ± 3.92[b]	<0.01	0.16	0.02

[a]Values are means ± SEM, n = 5. Means in the same row with superscripts without a common letter differ by Newman–Keuls multiple comparison test, $P < 0.05$.
[b]From two-way ANOVA.
[c]Fasting glucose (mM) × fasting serum insulin (ng/mL)/22.5.

and CLA-fed mice. However, both OC and EE were higher ($P < 0.05$) in CLA + Cr–fed mice than in SFO-fed mice. Analyzing the EE data by two-way ANOVA for main effects revealed statistically significant effects of CLA alone and Cr alone but not in combination (Table 4).

DISCUSSION

High-fat—diet–induced obesity is often associated with IR, which is a major risk factor for diabetes (49,50). Visceral obesity, in particular, has a strong correlation with IR and onset of glucose intolerance (51). Rats and mice fed a high-fat diet have increased VFM, whole body and muscle IR, and hyperinsulinemia within 4 wk, whereas continuous feeding of a high-fat diet for 8–12 wk develops severe visceral obesity, diabetes or impaired glucose tolerance, and plasma lipid and lipoprotein abnormalities (38). In the present study, CLA decreased body weight, BFM, and VFM, the last of which decreased further with the combination of CLA and Cr. Previous studies in animals fed CLA or the t10c12 isomer have shown decreased body weight and fat mass compared with control animals (40,52—54). Cr alone decreased body weight and fat mass in our study in accordance with previous studies (27). This is the first observation to show that Cr has beneficial effects on body composition in high-fat-diet–fed mice.

The antiadiposity effect of CLA has been ascribed, in part, to its ability to alter energy balance. Some studies in mice have shown that decreased energy intake may be insufficient to account for CLA—mediated dramatic loss of fat mass, implying that other mechanisms may be involved (40,41,52). Increased

TABLE 4
Oxygen Consumption and Energy Expenditure in Male Balb/c Mice Fed Control (SFO) or CLA Diets With or Without Chromium (Cr) Supplementation for 14 wk[a]

					P Value[b]		
	SFO	CLA	SFO + Cr	CLA + Cr	CLA	Cr	CLA × Cr
Energy expenditure (kcal/kg body weight)	11.29 ± 0.45[a]	13.11 ± 0.44[ab]	13.29 ± 0.54[ab]	14.15 ± 0.90[b]	0.04	0.03	0.44
Oxygen consumption (L O_2/kg body weight)	3.01 ± 0.12[a]	3.50 ± 0.12[ab]	3.54 ± 0.14[ab]	3.77 ± 0.24[b]	0.16	0.21	0.89

[a]Values are means ± SEM, n = 5. Means in the same row with superscripts without a common letter differ by Newman–Keuls multiple comparison test, $P < 0.05$.
[b]From two-way ANOVA.

EE has increasingly been proposed to be one of the mechanisms for the antiadiposity effects of CLA and particularly with the use of t10c12 isomer (40,41). Research suggests that uncoupling protein 2 (UCP2) expressed in white and brown adipose tissues may play a significant role in regulation of EE by CLA. UCP2 mRNA expression was shown to be augmented by CLA and t10c12 isomer in white adipose tissues from mice and rats, and in cultured adipocytes (21,22,52). We observed higher EE and OC in CLA + Cr–fed mice compared with control diet–fed mice, suggesting that CLA + Cr–mediated loss of fat mass may be associated, in part, with increased EE and OC.

Leptin, a product of the obese gene, is secreted primarily by adipocytes and plays an important role in regulating energy balance. Circulating leptin is highly correlated with general adiposity in obese rodents and individuals exhibiting higher plasma leptin levels (55,56)**.** Decreased leptin levels in CLA-fed mice were correlated with decrease in fat mass in the present study, suggesting that this may be one of the mechanisms involved in the observation. Because Cr did not lower circulating leptin levels in our study, Cr—induced loss of fat mass may be independent of leptin concentration. CLA and Cr have been reported to decrease serum leptin levels in animal studies (57,58). We have previously shown that CLA decreases leptin mRNA expression in peritoneal fat tissues, and CLA also decreases circulating leptin levels in high-fat-diet–fed mice (44).

In the present study, decreased glucose, insulin, and IRI were observed in CLA-fed mice, indicating a beneficial effect on IS. This is indeed an interesting finding considering that CLA and t10c12 isomer alone induced hyperinsulinemia, hyperglycemia, and IR in most of the studies in mice (21,22,59) and in humans (23–25). Studies suggest that PPAR-γ may be one of the key modulators of IR by CLA (more predominantly by the t10c12 isomer). PPAR-γ is downregulated in both adipose tissues from CLA-fed mice and cultured adipocytes. Downregulation of PPAR-γ contributes to loss of adiposity and decreased IS (21,60). Recently, Chung *et al*. found that t10c12 isomer decreases PPAR-γ expression in human adipocytes through NF-κB activation (26). This study provides evidence that inhibition of PPAR-γ in human adipocytes by CLA and/or t10c12 isomer may be a key mechanism involved in IR in humans. Interestingly, studies in normal and diabetic rats indicate beneficial effects of CLA and t10c12 isomer on glucose and insulin levels, glucose tolerance, and IS (52,61,62), which could be related to activation of PPAR-γ in these studies (61,63). Thus, the effect of CLA on IR seems to be dependent partly on species, metabolic state of the animal, and response of PPAR-γ to CLA, more specifically the t10c12 isomer. Wargent *et al*. recently demonstrated increased glucose and insulin levels, and exacerbated glucose tolerance, in genetically obese (lep^{ob}/lep^{ob}) mice fed CLA or t10c12 isomer–enriched CLA for 2 wk. However, long-term CLA intervention (10 wk) had beneficial effects on glucose, insulin, and IRI levels (64). This study and long-term studies in humans suggest that the detrimental effect of CLA on IR may be a transient effect seen in short-term studies; long-term studies did not show any adverse effects on glucose and insulin levels (19,20). However, these findings need to be confirmed with more long-term studies in rodents and humans using purified isomers. Cr alone significantly decreased insulin level and IRI in our study, in accordance with previous studies (34,57). Our results further suggest that CLA + Cr may have additive beneficial effects on insulin level and IRI in high-fat-diet–fed mice. Although the results from the present study are encouraging and suggest a beneficial effect of CLA + Cr on IS, more mechanistic studies in animal models need to be pursued to establish the efficacy of CLA + Cr supplementation on IR and body composition. Cr has been shown to activate insulin receptor kinase (IRK) but to downregulate insulin receptor tyrosine phosphatase (IRTP) (33). The activation of IRK and inhibition of IRTP may lead to increased phosphorylation of the insulin receptor and improved IS. Because PPAR-γ is an important target for CLA, studying the effect of Cr on PPAR-γ in adipocytes derived from rodents and humans may be of interest to establish the beneficial effect of Cr on IR in mice fed CLA.

Adiponectin is secreted by fat cells and enhances insulin action by suppressing hepatic glucose production and glucose uptake by skeletal muscles (65). In human studies, adiponectin levels are negatively correlated with fat mass (65,66). However, in a recent study in PPAR-γ adipose knockout mice, improved IS was observed along with decreased adipose tissue expression and lower plasma levels of adiponectin, suggesting that adiponectin produced by fat may not completely account for the improved IS (67). Moreover, Baratta *et al*. reported that the plasma adiponectin level was independent of fat mass in nondiabetic and diabetic patients (68). CLA and Cr alone or in combination had no effect on

serum adiponectin levels in the present study. Our previous study on the effects of CLA and exercise interaction in high-fat-diet–fed mice also did not show any effect on serum adiponectin levels or adiponectin mRNA expression in peritoneal fat pads derived from these mice (44). Adiponectin has been shown to be increased in CLA—fed diabetic rats but decreased in mice, which was associated with decreased IR (69—71). Improved IS without enhanced serum adiponectin levels in our study suggest that beneficial effects of CLA may be independent of adiponectin concentrations in this mouse model.

CLA alone and in interaction with Cr had beneficial effects on serum TNF-α and IL-6 levels in the present study. Our previous study in peritoneal fat derived from high-fat-diet–fed mice clearly indicated a statistically significant effect of CLA on TNF-α mRNA expression, which suggests that inhibition may occur at the adipose tissue level (44). CLA has been previously shown to decrease TNF-α and IL-6 in serum and macrophages derived from rats and mice (58,63,70,72), whereas other studies have reported either no effect or significant elevation of these cytokines in cultured cells from mice and humans (73,74). Cr has been reported to inhibit endotoxin—mediated inflammatory cytokine production in swine (75) and phytohemagglutinin—stimulated human peripheral blood mononuclear cells (76). The present data suggests that CLA and Cr may have additive effects in decreasing TNF-α and IL-6 in high-fat-diet–fed mice, which, in part, could explain improved IS in these mice.

We did not observe any enlarged livers in CLA—fed mice, although there was a dramatic decrease in fat mass. Previous studies show that CLA and t10c12 isomer increase liver weight in mice due to increased fat deposition (21,59). However, when CLA was supplemented with a high-fat diet, the mice did not show higher liver weight compared with control mice (77). A similar phenomenon may have influenced liver weights in this study.

In conclusion, the present study suggests that the combination of CLA and Cr lowers body weight and VFM, and may have a beneficial effect on IS in high-fat—diet–fed mice. More studies in different strains of mice and other species are warranted before the combination of CLA and Cr may be recommended to be beneficial in human health.

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Effect of Species and Genotype on the Efficiency of Enrichment of Poultry Meat with n-3 Polyunsaturated Fatty Acids

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ABSTRACT: The effect of poultry species (broiler or turkey) and genotype (Wrolstad or BUT T8 turkeys and Ross 308 or Cobb 500 broilers) on the efficiency with which dietary long-chain n-3 PUFA were incorporated into poultry meat was determined. Broilers and turkeys of both genotypes were fed one of six diets varying in FA composition (two replicates per genotype × diet interaction). Diets contained 50 g/kg added oil, which was either blended vegetable oil (control), or partially replaced with linseed oil (20 or 40 g/kg diet), fish oil (20 or 40 g/kg diet), or a mixture of the two (20 g linseed oil and 20 g fish oil/kg diet). Feeds and samples of skinless breast and thigh meat were analyzed for FA. Wrolstad dark meat was slightly more responsive than BUT T8 ($P = 0.046$) to increased dietary 18:3 concentrations (slopes of 0.570 and 0.465, respectively). The Ross 308 was also slightly more responsive than the Cobb 500 ($P = 0.002$) in this parameter (slopes of 0.557 and 0.449). There were no other significant differences between the genotypes. There was some evidence (based on the estimates of the slopes and their associated standard errors) that white turkey meat was more responsive than white chicken meat to 20:5 (slopes of 0.504 and 0.289 for turkeys and broilers, respectively). There was no relationship between dietary 18:3 n-3 content and meat 20:5 and 22:6 contents. If birds do convert 18:3 to higher FA, these acids are not then deposited in the edible tissues.

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The health benefits associated with increased consumption of EPA (C20:5) and DHA (C22:6) as found in fish oil are well established. A meta-analysis of 26 studies by Friedberg *et al.* in 1998 (1) demonstrated that supplementing the diet with fish oil reduced serum concentration of triacylglycerols, and this effect was even more marked in individuals with Type 2 diabetes. Increasing consumption of EPA and DHA would therefore contribute to a reduction in the incidence of metabolic syndrome, which is characterized by obesity, insulin resistance, or Type 2 diabetes and dyslipidemia (2). However, although recommended minimum intakes of EPA plus DHA are, for example, in the UK, 450 mg/d (3), actual mean consumption in the UK is apparently only about 244 mg/d. Closer analysis of dietary survey data (4) reveals that consumption is in fact lower still, at approximately 113 mg/d (5), as only 27% of the UK population consume any oily fish at all. In this scenario, poultry meat contributes approximately 23% of the EPA and DHA that is consumed (5). This pattern is also observed throughout northern Europe (6). Clearly, from a public health perspective, the consumption of EPA and DHA needs to increase, but efforts to encourage the consumption of oily fish or even fish oil capsules have largely failed. An alternative approach is to increase the EPA and DHA content of those foods (such as poultry meat) that are consumed and already contribute to EPA and DHA intake. The n-3 PUFA content of poultry meat can be readily increased by increasing the n-3 PUFA content of poultry diets (7–10). However, a review of several papers that reported such increases (11) noted that the response, particularly to EPA and DHA, was highly variable. Some of this variability may be due to differences in the species and genotype of bird used, but few data examining these relationships are available. The objective of this study was therefore to determine the effect of different species and genotypes of poultry on their response (as measured by increases in the n-3 PUFA content of their edible tissues) to increased concentrations of n-3 PUFA in their diet. Skinless meats were studied in this experiment, as these are more homogenous than meats with skin on, and also represent more closely what is actually consumed (at least in Europe and North America). Poultry meat is regarded as a low-fat food, and much of the fat that is present is trimmed off either before or after cooking. Thus by studying skinless meat, the impact on human nutrition of altering the FA composition of poultry tissues that are actually consumed could be determined.

EXPERIMENTAL PROCEDURES

Birds. The species and genotypes of poultry investigated were turkeys (using the bronze Wrolstad and white BUT T8 genotypes) and broilers (using the Ross 308 and Cobb 500 genotypes). These genotypes were selected to be indicative of those commonly consumed in Europe, except for the Wrolstad, which is reared for a more specialized niche Christmas market. Male turkey poults, 100, were purchased as day-old birds; half of them were Wrolstad (Kelly Turkeys, Danbury, Essex, United Kingdom) and the other half were BUT T8 (SCF Turkeys, Liverpool, United Kingdom). On arrival, they were brooded together for 8 wk in separate pens (divided by genotype) and fed a proprietary feed for turkey poults with clean, fresh water always available. Male broiler chicks, 120,

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Abbreviations: ALA, alpha-linolenic acid; LA, linoleic acid; LC n-3 PUFA, long-chain n-3 PUFA.

were also purchased as day-old birds (Anglia Chicks, Fakenham, Norfolk, United Kingdom); half of these were of the genotype Ross 308 and the other half were Cobb 500. On arrival they were brooded together for 3 wk in separate pens (again divided by genotype) and fed a proprietary chick crumb with clean, fresh water always available.

When the turkeys were 8 wk old and the broilers 3 wk old, they were randomly allocated to one of 24 pens (turkeys) or one of 24 cages (broilers). There were four turkeys per pen and six broilers per cage, and all the birds in one pen or cage were of the same genotype. The birds were then fed one of six experimental diets *ad libitum* with clean, fresh water always available. There were two replicates for each genotype × diet interaction. In the first week following allocation to the pens, the experimental diet was mixed on a 50:50 (fresh weight) basis with the proprietary starter pellet that the birds had been fed prior to allocation to the pens.

When the broilers were 42 d old and the turkeys 16 wk old, they were humanely slaughtered, bled, plucked, and eviscerated. The head and feet were removed and carcasses were then stored at <5°C in labeled plastic bags. After 7 d, one carcass from each pen was carefully dissected to separate the breast and thigh meat. Samples of skinless breast and thigh meat were placed in clean labeled plastic bags and stored at –20°C pending analysis.

Diets. Six different diets were fed to the birds, varying in their proportion of α-linolenic acid (ALA), EPA, and DHA. All diets contained 70 g/kg (fresh weight) ether extract, with 50 g of this ether extract coming from added oil. In the control diets, all the oil was in the form of blended vegetable oil. In diets Lolin and Hilin, the vegetable oil was partially substituted with linseed oil, such that the diets contained 20 and 40 g/kg (fresh weight) linseed oil respectively. In diets Lofish and Hifish, the vegetable oil was partially substituted with fish oil, such that the diets contained 20 and 40 g/kg (fresh weight) fish oil respectively. In diet Linfish, the diet contained 20 g/kg linseed oil and 20 g/kg fish oil (fresh weight basis). Details of the composition of the diets used, and their calculated nutrient compositions, are provided in Tables 1 and 2. The antioxidant used was predominantly vitamin E, which was included in all diets at a rate of 100 mg DL-α-tocopherol acetate/kg diet as fed. In the turkey diets, BHT was also included in the vitamin/mineral mix that was used, and was present in the diets at a concentration of 20 mg/kg (as fed).

Analysis. The feeds and the samples of white and dark meat taken from the birds were analyzed for long-chain FA. Lipids were extracted from samples of feed (3 g) or grated frozen meat tissue (20 g) using the method of Folch *et al*. (12). Samples were then methylated by drying the lipid extract under a stream of nitrogen and then dissolving the residue in dried toluene (1 mL) and sodium methoxide (2.7 g dry sodium methoxide in 100 mL methanol, 2 mL). Glacial acetic acid (0.1 mL) and hexane (2 mL) were added and mixed, followed by water (5 mL). The hexane layer containing the FAME was then separated off and analyzed using a gas chromatograph-mass spectrometer (Hewlett Packard GCD Series 2). Samples were injected (split injection) onto a capillary column (60 m, 0.32 mm diameter) with helium (flow rate 1 mL/min) used as the carrier gas. The column was held at 70°C for 2 min before being raised to 165°C (5°C/min) for 5 min. It was then raised to 180°C (6°C/min) for 10 min and then 230°C (3°C/min) for 3 min. The inlet temperature was 260°C and the detector temperature 250°C. FAME were identified by comparing retention times with those of standards and quantification was done by normalizing the areas under the curve. FA concentrations were calculated in terms of % wt/wt total FA detected. Mean concentrations (mg/100 g fresh meat tissue) of total FA were calculated for each of the four meats (white chicken, dark chicken, white turkey, and dark turkey). From these estimates, the concentrations of individual FA in each poultry meat were calculated and expressed as mg/100 g edible portion.

Statistical analysis. ANOVA was used to determine the ef-

TABLE 1
Composition of the Diets (g/kg Fresh Weight) Fed to Broilers and Turkeys

	Broiler	Turkeys, 8–12 wk	Turkeys, 13–16 wk
Wheat	416	400	422
Soybean meal	300	300	300
Wheatfeed[a]	50	50	50
Sunflower meal	41	44	40
Beans (*Phaseolus vulgaris*)	50	50	50
Fishmeal	50	50	50
Oil (vegetable, fish, or linseed)	50	50	50
Dicalcium phosphate	20.8	26	18
Calcium carbonate	15	20	12
Salt	2.2	2.5	2.6
dl-Methionine	0	1.2	0.4
l-Lysine	0	1.3	0
Vitamin/mineral premix	5	5	5
Premix provided (per kg diet as fed):			
Vitamin A (IU)	12,000	15,000	12,000
Vitamin D3 (IU)	5,000	5,000	4,000
Vitamin E (mg)	100	100	100
Copper (mg)	20	20	20
Zinc (mg)	80	100	80
Choline chloride (mg)	200	—	250

[a]Wheatfeed is a coproduct of milled wheat comprising a mixture of the bran and endosperm that is separated when cleaned and dehusked grain is milled for flour.

TABLE 2
Calculated Nutrient Contents of the Diets Fed to Broilers and Turkeys (kg^{-1} as Fed)

	Broiler	Turkeys, 8–12 wk	Turkeys, 13–16 wk
Metabolizable energy, kcal	3,250	3,203	3,274
Crude protein, g	262	261	262
Lysine, g	14.1	15.4	14.1
Methionine, g	5.0	6.2	5.4
Methionine + cystine, g	19.1	20.3	19.5
Tryptophan, g	4.0	3.9	4.0
Threonine, g	10.5	10.5	10.5
Arginine, g	18.5	18.5	18.5

fect of genotype, diet, and the interaction between genotype and diet on the FA content of the different poultry tissues. The FA contents of white and dark meat were compared using a paired student's t-test. To consider the efficiency with which FA were enriched in poultry meat, a linear model was used to test the hypothesis of a straight-line relationship between the concentration of an individual FA in the diet (% wt/wt total FA) and its concentration (% wt/wt total FA) in edible tissue (either white or dark meat). The interaction between this estimate of slope and the genotype of the bird was also examined. A linear mixed model was then used to determine whether there was a significant difference between tissues (and a significant interaction with genotype of bird) in the response to increased dietary concentrations of an individual FA. Statistical comparisons were made using SAS (SAS Institute Inc., Cary, NC). In the case of turkeys, the concentrations of dietary FA used were those estimated in the diets fed to the turkeys from 13 to 16 wk of age. No direct statistical comparisons between species of birds could be made, as they were reared for different times, in different houses, and on slightly different diets. However, the slopes and standard errors of the responses to increased dietary concentrations of FA were compared to determine whether there was any evidence of a difference between species in their response to dietary FA concentrations.

To ascertain whether there was any difference between genotypes in increased meat concentrations of EPA and DHA resulting from an increased supply of dietary ALA, the linear relationship between diet ALA content and meat EPA or DHA content was estimated using a linear model for each tissue. Data from birds fed Lofish, Hifish, or Linfish were excluded from this analysis as the EPA and DHA present in these diets would have confounded the estimation.

RESULTS

The concentrations of FA in the different diets are presented in Table 3. Increasing the linseed oil content of the diet increased the ALA content of the diet. Adding fish oil to the diet increased the EPA and DHA content of the diet but had a negligible effect on the ALA content. Compared with the controls, all diets had lower concentrations of 18:2 (LA), and lower ratios of n-6:n-3 FA.

TABLE 3
FA Composition (% wt/wt Total FA) of Diets Used

Diet	16:0	18:′0	18:1 n-9	18:2 n-6	18:3 n-3	20:4 n-6	20:5 n-3	22:6 n-3
Broilers, 3–6 wk								
Control	12.2	4.2	20.5	54.0	7.1	0.06	0.73	1.34
Lolin	11.2	4.1	19.3	42.4	21.0	0.06	0.71	1.2
Hilin	9.3	3.5	18.7	31.8	34.0	0.19	0.85	1.8
Lofish	14.4	4.0	20.6	43.2	6.6	0.38	4.0	6.9
Hifish	18.0	3.8	20.7	32.3	5.1	0.66	7.4	12.0
Linfish	12.3	3.6	19.4	31.0	22.2	0.31	4.07	7.1
Turkeys, 8–12 wk								
Control	13.3	3.0	21.5	52.5	7.2	0.03	0.83	1.7
Lolin	10.8	3.2	20.8	43.5	19.8	0.03	0.69	1.2
Hilin	9.7	3.1	19.9	35.5	29.4	0.02	0.85	1.5
Lofish	14.7	3.4	23.2	44.3	7.1	0.39	3.0	3.9
Hifish	18.0	3.6	22.2	36.1	4.6	0.61	5.7	9.2
Linfish	12.2	3.3	20.0	31.4	21.9	0.02	4.0	7.2
Turkeys, 13–16 wk								
Control	13.3	3.7	20.7	52.8	7.3	0.04	0.73	1.4
Lolin	11.0	3.2	20.1	44.1	19.1	0.03	0.57	0.99
Hilin	10.2	3.2	19.6	36.3	28.5	0.06	0.79	1.4
Lofish	14.7	3.4	23.2	44.3	7.1	0.39	3.0	3.9
Hifish	17.0	3.6	21.5	37.5	5.3	0.51	5.5	9.1
Linfish	12.6	3.3	20.1	33.6	21.9	0.10	3.1	5.3

FA content of edible tissues. Regarding the concentrations of FA in both white and dark meat, there were no significant interactions (with either broilers or turkeys), between diet and the genotype of bird for the concentrations of FA in either white or dark meat (Tables 4–7). The Lofish and Hifish diets increased the 16:0 content of both white and dark meat. The 18:1 n-9 content of Wrolstad dark meat was approximately 12% greater ($P = 0.044$) than that of BUT T8 dark meat (Table 7).

The LA content of meat was not affected by the genotype of bird (be it turkey or broiler), but there was a significant reduction in LA content as vegetable oil was replaced by either linseed oil or fish oil, except in the case of turkey white meat. Conversely, there was a highly significant ($P < 0.001$) increase in ALA content of all meats when linseed oil was included in the poultry diets, with the genotype of bird having

TABLE 4
Effect of Breed and Diet on the Mean FA Content (mg/100 g Tissue) in Chicken White Meat[a]

	Control		Lolin		Hilin		Lofish		Hifish		Linfish			P^b		
FA	Ross	Cobb	Ross	Cobb	Ross	Cobb	Ross	Cobb	Ross	Cobb	Ross	Cobb	SE	B	D	B × D
16:0	233	227	204	227	214	220	241	243	285	247	220	228	11.3	0.910	0.006	0.206
18:0	0.9	0.9	1.1	0.7	1.2	0.8	1.5	0.9	0.8	0.8	1.3	0.9	0.48	0.005	0.190	0.439
18:1 n-9	230	243	242	237	267	232	253	270	238	229	239	222	17.9	0.578	0.537	0.724
18:2 n-6	316	309	298	263	218	223	282	242	170	206	239	215	12.4	0.153	0.000	0.081
18:3 n-3	28.1	31.9	118	80.8	147	131	35.4	33.8	18.3	23.5	124	96.7	14.92	0.182	0.000	0.641
20:4 n-6	50.6	49.0	23.4	40.5	20.3	31.9	22.9	33.3	25.1	28.5	18.4	30.7	5.29	0.013	0.004	0.564
20:5 n-3	7.5	6.9	10.4	5.1	13.5	20.0	17.4	20.0	27.2	30.8	22.0	25.9	2.60	0.258	0.000	0.326
22:6 n-3	39.6	38.6	29.7	51.6	31.3	48.3	54.9	64.3	118	126	47.9	83.8	12.24	0.053	0.000	0.737

[a]Diets comprise (g/kg diet); Control, 50 vegetable oil (VO); Lolin, 20 linseed oil (LO), 30 VO; Hilin, 40 LO, 10 VO; Lofish, 20 fish oil (FO), 30 VO; Hifish, 40 FO, 10 VO; Linfish, 20 LO, 20 FO, 10 VO. Ross, Ross 308; Cobb, Cobb 500.
[b]B, breed of bird; D, diet; B × D, interaction between breed and diet.

TABLE 5
Effect of Breed and Diet on the Mean FA Content (mg/100 g Tissue) in Chicken Dark Meat[a]

	Control		Lolin		Hilin		Lofish		Hifish		Linfish			*P*		
FA	Ross	Cobb	Ross	Cobb	Ross	Cobb	Ross	Cobb	Ross	Cobb	Ross	Cobb	SE	B	D	B × D
16:0	285	298	268	287	256	275	321	339	335	334	288	300	6.6	0.007	0.000	0.679
18:0	109	115	96	110	111	120	133	103	116	130	117	112	10.9	0.908	0.600	0.370
18:1 n-9	400	393	420	391	384	375	354	450	461	407	405	387	26.8	0.842	0.582	0.180
18:2 n-6	468	457	402	379	322	313	377	367	318	323	323	322	11.8	0.259	0.000	0.888
18:3 n-3	56.3	57.6	161	155	258	209	49.5	59.2	38.7	47.1	179	167	6.11	0.150	0.000	0.014
20:4 n-6	29.4	27.8	13.0	22.6	15.6	30.4	36.4	13.6	13.8	21.3	13.4	19.2	6.41	0.706	0.393	0.151
20:5 n-3	5.0	7.3	5.8	10.0	10.8	21.4	24.7	19.0	35.0	40.2	22.8	25.7	2.92	0.131	0.000	0.290
22:6 n-3	19.9	21.0	12.1	20.0	17.5	28.6	76.2	31.7	69.9	88.0	35.3	48.8	9.53	0.914	0.000	0.065

[a]For diet oil content and abbreviations, see Table 4.

TABLE 6
Effect of Breed and Diet on the Mean FA Content (mg/100 g Tissue) in Turkey White Meat[a]

	Control		Lolin		Hilin		Lofish		Hifish		Linfish			*P*		
FA	Wrol	BUT	Wrol	BUT	Wrol	BUT	Wrol	BUT	Wrol	BUT	Wrol	BUT	SE	B	D	B × D
16:0	204	194	177	180	205	169	195	195	214	218	189	198	9.8	0.356	0.047	0.329
18:0	113	114	87.8	82.3	95.5	90.8	107	98.9	119	97.5	124	108	11.27	0.225	0.146	0.934
18:1 n-9	163	152	170	181	173	175	179	167	152	163	170	171	15.4	0.986	0.708	0.944
18:2 n-6	227	267	277	280	233	251	260	255	221	234	207	210	19.6	0.317	0.072	0.884
18:3 n-3	75.0	22.0	107	117	124	128	18.3	31.9	21.0	37.0	69.6	68.5	22.20	0.903	0.003	0.637
20:4 n-6	46.5	73.6	27.4	16.2	20.4	27.1	36.9	46.3	35.1	25.0	31.4	32.5	11.75	0.579	0.065	0.619
20:5 n-3	10.8	10.6	8.0	7.7	9.5	11.3	19.4	18.1	30.4	29.8	25.7	26.9	3.27	0.992	0.000	0.997
22:6 n-3	42.5	49.5	27.2	18.4	22.1	29.5	66.5	70.2	90.2	78.5	65.0	67.4	12.20	0.982	0.002	0.943

[a]Wrol, Wrolstad; BUT, BUT T8. For diet oil content and other abbreviations, see Table 4.

TABLE 7
Effect of Breed and Diet on the Mean FA Content (mg/100 g Tissue) in Turkey Dark Meat[a]

	Control		Lolin		Hilin		Lofish		Hifish		Linfish			*P*		
FA	Wrol	BUT	Wrol	BUT	Wrol	BUT	Wrol	BUT	Wrol	BUT	Wrol	BUT	SE	B	D	B × D
16:0	435	415	370	416	382	410	442	435	443	495	394	422	22.7	0.130	0.043	0.568
18:0	242	249	196	216	194	234	209	219	206	219	206	225	19.3	0.125	0.433	0.958
18:1 n-9	342	285	320	331	327	299	365	318	375	300	353	331	28.0	0.044	0.798	0.721
18:2 n-6	538	576	533	489	457	427	514	557	441	455	436	423	30.2	0.941	0.004	0.624
18:3 n-3	55.7	61.4	206	174	285	250	76.3	73.0	61.2	64.1	213	186	14.98	0.109	0.000	0.579
20:4 n-6	80.8	80.9	63.5	62.7	49.7	62.9	46.7	55.9	45.5	50.7	37.9	46.6	12.35	0.420	0.094	0.991
20:5 n-3	16.8	24.8	21.0	21.5	26.8	29.8	33.0	29.4	60.3	54.2	42.1	44.1	4.76	0.829	0.000	0.737
22:6 n-3	59.7	77.2	60.8	60.3	48.3	57.9	84.3	82.4	139	131	86.7	93.0	17.26	0.707	0.006	0.979

[a]For diet oil content and abbreviations, see Tables 4 and 6.

no significant effect. Replacing vegetable oil with n-3–rich oils reduced the 20:4 content of white chicken meat (P = 0.004), and Ross 308 white meat had a lower concentration of 20:4 than Cobb 500 white meat (P = 0.013).

With regard to the long–chain (LC) n-3 PUFA, increasing the concentration of fish oil in the diet resulted in significant increases in the concentration of both EPA and DHA in all meats. White Cobb 500 meat tended (P = 0.053) to have a higher concentration of DHA than Ross 308 meat, but otherwise there was no effect of poultry genotype. The EPA content of turkey meat was increased by a factor of approximately 2.8 when Hifish was fed instead of control, and the DHA content was also nearly doubled. The DHA content of white chicken meat was trebled, and that of dark chicken meat nearly quadrupled by feeding Hifish instead of control. The most dramatic increases were observed with the EPA content of chicken meat, however, which was increased by a factor of six in the dark meat when Hifish was fed instead of control. The amount of EPA + DHA that might be consumed by eating 100 g portions of poultry fed with Hifish compared with control was doubled in turkeys (from 57 to 114 mg in white meat, and from 89 to 192 mg in dark meat). In broilers, the increase was 226% in white meat (from 46 to 151 mg) and 340% in dark meat (from 27 to 117 mg).

The FA content of dark meat was in all cases greater than that of white meat ($P < 0.001$), except for EPA in chicken meat, in which there was no significant difference, and for 20:4 and DHA in chicken meat, in which the white meat was a richer source than dark meat.

Response to dietary concentrations of LNA, EPA, and DHA. Increasing the dietary concentration of LNA, EPA, or DHA resulted in significant ($P < 0.001$) linear increases in the concentration (% wt/wt total FA) of these acids in poultry meat (Table 8). Generally, there was no significant difference

TABLE 8
Effect of Poultry Genotype on the Linear Relationship Between the Concentrations of Meat and Diet ALA, EPA, and DHA

	Turkey genotype			P^a		Broiler genotype			P^a	
FA	Wrol	BUT	SE	L	L × B	Ross	Cobb	SE	L	L × B
White meat										
ALA	0.481	0.447	0.125	0.000	0.847	0.474	0.368	0.052	0.000	0.164
EPA	0.498	0.510	0.082	0.000	0.919	0.257	0.320	0.059	0.000	0.461
DHA	0.728	0.884	0.203	0.000	0.591	0.682	0.690	0.128	0.000	0.967
Dark meat										
ALA	0.570	0.465	0.035	0.000	0.046	0.557	0.449	0.022	0.000	0.002
EPA	0.332	0.444	0.064	0.000	0.229	0.317	0.307	0.041	0.000	0.869
DHA	0.465	0.573	0.114	0.000	0.514	0.396	0.406	0.086	0.000	0.937

[a]L, significance of the linear relationship between diet and meat n-3 PUFA content; L × B, significance of the interaction between L and the genotype of bird. For other abbreviations, see Tables 4 and 6.

between the two turkey genotypes in their response to these acids, but Wrolstads did show a greater response to ALA in their dark meat compared with BUT T8. Similarly, the only significant difference between broiler genotypes in their response to these FA was the response in dark meat to ALA, with Ross 308 being more responsive than Cobb 500. There was no significant difference between white and dark meat in terms of its responsiveness to increased concentrations of dietary ALA or EPA in turkeys, but there was a tendency (P = 0.079) for white meat to be more responsive than dark meat to DHA. In broilers, there was a tendency (P = 0.078) for dark meat to be more responsive than white meat to ALA, and although there was no significant difference in their responsiveness to EPA, the response to DHA was much greater (P = 0.001) in white meat compared with dark meat. There was no difference between the broiler genotypes in this differential response by different tissues. Based on the evidence of the coefficients and their standard errors, there was some evidence that turkey white meat may be more responsive than broiler white meat to increased dietary concentrations of EPA, but otherwise there was no evidence of any difference in efficiency between the two species.

There was no evidence of a linear relationship between dietary ALA content and the concentration of EPA or DHA in the meat (Table 9). There was also no difference between either broiler or turkey genotypes in this parameter, and no evidence of a difference between the species of poultry. In all cases, the coefficient describing the relationship was ≤0.1, and in some cases was negative.

DISCUSSION

Enrichment of poultry meat with n-3 PUFA. The levels of enrichment observed in this experiment with white chicken meat were similar to those reported when broilers were fed a stabilized fishmeal formulation comprising 200 g/kg of the diet (13) or a diet comprising 120 g/kg redfish meal (8). For dark meat, our results were very similar to those observed from feeding 120 g/kg redfish meal (8) or 40 g/kg fish oil (14) to broilers. However, the experiment reported by Howe *et al*. in 2002 (13) achieved much greater enrichment of dark meat by feeding a stabilized fishmeal formulation, and our results were equivalent to their feeding just 50 g/kg of this formulation. Much greater degrees of enrichment in white and dark meat were reported by Gonzalez-Esquerra and Leeson (15) when they fed broilers a diet comprising just 15 g/kg menhaden oil, but in this case the FA content was measured in cooked meat and Howe *et al*. (13) demonstrated that cooking can more than double the FA content in the tissue, presumably because of moisture loss. The LC n-3 PUFA content observed by Gonzalez-Esquerra and Leeson (15) is therefore likely, in the fresh tissue, to be similar to that observed in this experiment, although it is remarkable that such a high degree of enrichment could be obtained by them with such a low level of inclusion of the fish oil in the diet. This serves to illustrate the variability in the LC n-3 PUFA content of different fish oils, and does explain, in part, the variability in enrichment observed when similar quantities of fish oil are fed. For example, Zanini *et al*., in 2004, fed broilers diets com-

TABLE 9
Effect of Poultry Genotype on the Linear Relationship Between Diet ALA Content and Meat EPA or DHA Content

	Turkey genotype			P^a		Broiler genotype			P^a	
Acid	Wrol	BUT	SEM	L	L × B	Ross	Cobb	SEM	L	L × B
White meat										
EPA	–0.008	0.003	0.017	0.829	0.667	0.022	0.004	0.031	0.575	0.689
DHA	–0.110	–0.115	0.078	0.075	0.968	–0.031	–0.185	0.106	0.187	0.334
Dark meat										
EPA	0.026	0.012	0.016	0.119	0.532	0.016	–0.019	0.030	0.947	0.413
DHA	–0.029	–0.053	0.029	0.082	0.578	–0.007	–0.129	0.071	0.250	0.231

[a]For abbreviations, see Tables 4, 6, and 7.

prising 60 g/kg fish oil yet only achieved LC n-3 PUFA contents of 54 mg/100 g in white meat and 42 mg/100 g in dark meat (16). Similarly, feeding broilers diets comprising 55 g/kg marine algae resulted in the LC n-3 PUFA content of the white meat being just 20 mg/100 g (17).

Although conventional poultry meat contributes relatively little LC n-3 PUFA (a 100-g serving of the meat produced in this study would contribute just 12% of the recommended minimum daily intake), poultry meat enriched by dietary means with LC n-3 PUFA does result in meat with a nutritionally meaningful LC n-3 PUFA content. In this experiment, 100 g servings of either chicken or turkey meat (be it white or dark) would contribute approximately 32% of the recommended minimum daily intake of 450 mg LC n-3 PUFA.

Effect of poultry species and genotype on enrichment efficiency. The results of this experiment demonstrated that, in modern broiler genotypes, there was no significant difference in the efficiency with which LC n-3 PUFA were incorporated into edible tissue. Even the more genetically diverse white and bronze genotypes of turkey showed a difference only in the incorporation of ALA into dark meat (as did the broilers). This limited difference may still suggest that there is some scope for selecting birds on the basis of their efficiency of transferring dietary n-3 PUFA to their meat tissues, but such selection cannot be at the cost of meat growth potential if this approach is to be viable. There is little evidence from this experiment (and from comparing data from the literature) to suggest that there is any inherent difference between broilers and turkeys in their ability to incorporate n-3 PUFA in their edible tissues, except perhaps for EPA in white meat.

The evidence that white chicken meat is a richer source of DHA than dark meat and also has a greater enrichment efficiency than dark meat for DHA is promising, particularly from the European Union viewpoint, since the consumption of white poultry meat far outweighs the consumption of dark meat in the EU. It is not a surprising finding, as the LC n-3 PUFA preferentially accumulate in the phospholipids, which are much more prevalent in the white meat compared with the dark meat (7).

The enrichment of EPA and DHA that was achieved in the white and dark meat with the Hifish diet amounted to (mean wt/wt total FA) 3.1% EPA and 8.8% DHA. It is not known what the maximum possible degree of EPA and DHA enrichment in poultry meat is, but it is perhaps worth noting that the skeletal muscle of the king penguin chick, which is hatched from eggs containing the highest known DHA content and which then feeds exclusively on fish, was quite refractory to changes in dietary EPA and DHA content (18). Indeed, although the EPA and DHA content of adipose tissue in the first month of growth increased rapidly and by the time of thermal emancipation was essentially identical to that of the diet, the DHA content of muscle phospholipid in particular remained remarkably constant at about 11.5% (18). There may of course be species differences, but these data may suggest that the maximum degree of enrichment of DHA that may be possible in poultry meat is around 12% wt/wt total FA. This was the degree of enrichment achieved in white chicken meat with the Hifish diet, and would be equivalent to approximately 113 mg DHA/100 g white poultry meat and 189 mg DHA/100 g dark poultry meat.

Enrichment of poultry meat with EPA and DHA in response to dietary LNA. If sources other than marine oils are to be used to enrich poultry meats with LC n-3 PUFA, then a disappointing result was the lack of relationship between the dietary ALA content and the EPA and DHA content of the meat. This relationship was calculated after data that involved feeding EPA and DHA to poultry were excluded. It therefore examined the birds' ability to convert dietary ALA to EPA and DHA and then deposit these LC n-3 PUFA in the edible tissues. The evidence from this experiment is that such a process is extremely limited, and this was also the conclusion of López-Ferrer *et al*. in 2001 (19). Their work suggested that although the birds may be capable of converting ALA to EPA and DHA to some extent, these acids were not then deposited in skeletal muscle but rather sequestered in the liver or transported to other tissues. It is not clear what mechanisms control the transport and distribution of EPA and DHA within the bird, but developing a means of controlling and manipulating such pathways would be an extremely valuable means of increasing the impact of measures to enrich poultry meat with the nutritionally valuable LC n-3 PUFA, without reliance on fish oils.

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Dose-Response Impact of Various Tocotrienols on Serum Lipid Parameters in 5-Week-Old Female Chickens

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ABSTRACT: The cholesterol-suppressive action of the tocotrienol-rich-fraction (TRF) of palm oil may be due to the effect of its constituent tocotrienols on β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase activity. The tocotrienols modulate HMG-CoA reductase activity via a post-transcriptional mechanism. As a consequence small doses (5–200 ppm) of TRF-supplemented diets fed to experimental animals lower serum cholesterol levels. These findings led us to evaluate the safety and efficacy of large supplements of TRF and its constituents. Diets supplemented with 50,100, 250, 500, 1,000, or 2,000 ppm of TRF, α-tocopherol, α-tocotrienol, γ-tocotrienol, or δ-tocotrienol were fed to chickens for 4 wk. There were no differences between groups or within groups in weight gain, or in feed consumption at the termination of the feeding period. Supplemental TRF produced a dose-response (50–2,000 ppm) lowering of serum total and LDL cholesterol levels of 22% and 52% ($P < 0.05$), respectively, compared with the control group. α-Tocopherol did not affect total or LDL-cholesterol levels. Supplemental α-tocotrienol within the 50–500 ppm range produced a dose-response lowering of total (17%) and LDL (33%) cholesterol levels. The more potent γ and δ isomers yielded dose-response (50–2,000 ppm) reductions of serum total (32%) and LDL (66%) cholesterol levels. HDL cholesterol levels were minimally impacted by the tocotrienols; as a result, the HDL/LDL cholesterol ratios were markedly improved (123–150%) by the supplements. Serum triglyceride levels were significantly lower in sera of pullets receiving the higher supplements. The safe dose of various tocotrienols for human consumption might be 200–1,000 mg/d based on this study.

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Hypercholesterolemia and inflammation of coronary arteries are major factors in the development of coronary heart disease in humans (1,2). Recently, inflammation has been implicated in the pathogenesis of atherosclerosis, cancer, stroke, and diabetes in human subjects (3–5). The major risk factors for atherosclerosis are elevated levels of serum total cholesterol and LDL cholesterol and inflammation of coronary arteries caused by increased levels of C-reactive protein (CRP; 6–9). CRP is now considered the best indicator for the risk of coronary heart disease (6,7). However, physiological and genetic factors also contribute to the progression of heart disease (10). The treatment of hypercholesterolemia with exercise, diet, and lipid-lowering agents has been the mainstay for treatment of atherosclerosis (11–15).

Dietary modifications recommended for the modulation of serum cholesterol levels are generally based on approaches that reduce cholesterol and saturated FA intake, reduce cholesterol absorption, and increase the excretion of cholesterol metabolites (16). Our research has roots in the anomalous finding that animals fed palm oil, a tropical oil rich in palmitic acid, had lower than predicted serum cholesterol levels (17,18). Our subsequent studies have identified tocotrienol-rich fraction (TRF) of palm oil as the source responsible for lowering serum cholesterol levels in experimental animal models (19). TRF consists of a mixture of α-tocopherol, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol. However, only the unsaturated analogues, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol, are responsible for hypocholesterolemic properties (20,21). The tocotrienols, with varying levels of efficacy, regulate cholesterol synthesis via a post-transcriptional mechanism, which leads to down-regulation of β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase activity (22). Mevalonic acid, the product of this reaction, is considered to be the rate-limiting substrate for the synthesis of cholesterol in the body (23). A decrease in serum total cholesterol, a response consistent with the tocotrienol-mediated down-regulation of HMG-CoA reductase activity, has been reported by a number of other investigators (24–28).

We have recently reported a number of biological activities of tocotrienols, which interfere with the formation of atherosclerotic plaque, including hypercholesterolemic, antioxidant, anti-inflammatory, and antiproliferation properties (29). Tocopherols, as well as tocotrienols, are classified as "generally regarded as safe" (GRAS) compounds by the U.S. Food and Drug Administration in small doses for human consumption and are remarkably free of any adverse effects (30). In earlier studies, experimental animal models were fed respective commercial diets supplemented with smaller doses of 5–200 ppm α-tocopherol, α-tocotrienol, γ-tocotrienol, δ-tocotrienol, or TRF for 4–6 wk (24–29,31,32). The effects of feeding higher levels of TRF or tocotrienols (50–2,000 ppm) on body weight gain and serum lipid parameters have not previously been determined. However, weight gain by male Fis-

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Abbreviations: α-TTP, α-tocopherol transfer protein; CRP, C-reactive protein; HMG-CoA, β-hydroxy-β-methylglutaryl coenzyme A; TRF, tocotrienol-rich fraction.

cher 344 rats was suppressed by feeding a diet supplemented with 4,000 ppm of α-tocotrienol (33).

In order to determine the safety and efficacy of TRF and its constituent tocols (mixture of tocopherol plus tocotrienols), the present study was carried out to examine the effects of large dietary supplementation of TRF, α-tocopherol, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol on weight gain, organ weights (liver, lungs, pancreas, heart, and kidneys), and serum lipid parameters (total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides) of chickens fed diets supplemented with 50, 100, 250, 500, 1,000, or 2,000 ppm of the aforementioned agents for 4 wk. Earlier findings show that the lipogenic activities of avians (34) and humans (35) are predominantly located in the liver, whereas the lipogenic activities of rodents are shared between liver and adipose tissue. Moreover, the levels of HDL and LDL cholesterol are similar in avians and humans (1.3 and 3.9 mmol/L for HDL and LDL, respectively), but differ in rodents (4.1 and 1.4 mmol/L for HDL and LDL, respectively). These factors led us to employ chickens for the present study (34,35). The young chickens (1 wk old) were chosen for the present study due to lack of pure large quantities of individual tocotrienols.

EXPERIMENTAL PROCEDURES

Materials. Sources of all chemicals, substrates, and diagnostic kits have been identified previously (29,31,32). Chemicals and solvents were of analytical grade. The commercially available TRF isolated from palm oil is a blend of α-tocopherol (25%), α-tocotrienol (25%), γ-tocotrienol (25%) and δ-tocotrienol (5%). After purification, in our laboratory, the TFR contained α-tocopherol (16.8%), α-tocotrienol (26.5%), γ-tocotrienol (42.5%) and δ-tocotrienol (14.1%). The ratio of tocotrienols vs tocopherols is important for the effects observed with tocotrienols, and these ratios can vary significantly from 1–5 in various preparations of TRF. Purified α-tocopherol, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol were provided by Palm Oil Research Institute of Malaysia (PORIM, Kuala Lumpur, Malaysia). The individual components of TRF were purified as described in detail in our recent paper (29). The purities of α-tocopherol and individual tocotrienols were established by HPLC and MS as reported earlier (29). The tocols of corn oil were removed by extracting the oil with ethyl alcohol.

Effects of different doses of tocotrienols on gain in body weight and lipid parameters in 5-wk-old female chickens. White Leghorn 1-wk-old female chickens were supplied by the Poultry Research Laboratory, University of Wisconsin—Madison, and weighed 40–50 g. The chickens were divided into 34 groups of 6 each. Each group was housed in a single brooder at the University of Wisconsin—Madison's Poultry Research Laboratory with 24 h light and free access to water and a cholesterol-free corn–soy meal diet for a 4-wk period. The control group for each series of treatments was fed the unsupplemented diet for 4 wk.

The diet consists of corn (8.8% protein, 615 g), soybean meal (44% protein, 335 g), tocol-stripped corn oil (10 g), calcium carbonate (10 g), dicalcium phosphate (20 g), iodized salt (5 g), and mineral and vitamin mixtures (2.5 g of each), which provided the following per kg feed: zinc sulfate·H_2O, 110 mg; manganese sulfate·$5H_2O$, 70 mg; ferric citrate·H_2O, 500 mg; copper sulfate·$5H_2O$, 16 mg; sodium selenite, 0.2 mg; DL-methionine, 2.5 mg; choline chloride (50%), 1.5 g; ethoxyquin, 125 mg; thiamine HCl, 1.8 mg; vitamin A, 1,500 IU; vitamin D3, 400 IU; vitamin E (D-tocols of corn, a mixture of 12% α-tocopherol, 3% α-tocotrienol, 7% β-tocotrienol, 70% γ-tocopherol, 6% γ-tocotrienol, and 2% δ-tocopherol), 10 IU (10 ppm, 23 μmol); riboflavin, 3.6 mg; calcium pantothenate, 10 mg; niacin 25 mg; pyridoxine HCl, 3 mg; folacin, 0.55 mg; biotin, 0.15 mg; vitamin B_{12} 0.01 mg; and vitamin K, 0.55 mg. Six experimental groups were fed diets supplemented with 50, 100, 250, 500, 1,000, or 2,000 ppm of the TRF isolated from palm oil. TRF consists of a blend of α-tocopherol (16.8%), α-tocotrienol (26.5%), γ-tocotrienol (42.0%), and δ-tocotrienol (14.1%). The tocotrienols account for 82.6% of the TRF. Diets for the remaining experimental groups were supplemented with 115 (50 ppm), 230 (100 ppm), 575 (250 ppm), 1,150 (500 ppm), 2,300 (1,000 ppm), or 4,600 (2,000 ppm) μmol of α-tocopherol, α-tocotrienol, γ-tocotrienol, or δ-tocotrienol per kg diet.

The required amount of each tocol or TRF was dissolved in hexane (50 mL) and mixed with the commercial diet (10 kg) for 30 min permitting the evaporation of hexane. The experimental diets were stored at 4°C throughout the feeding period.

Chickens and diets were weighed at the start and end of the 4-wk test. At the end of the feeding period, the birds were fasted for 12 h prior to sacrifice to facilitate chylomicron and VLDL clearance. The birds were sacrificed in three sets, each having its own control, by severing their carotid arteries. The collected sera were stored at –20°C for pending analyses. Organs were removed, blotted, and weighed. Feed consumption and efficiency were calculated from body and feed weights taken at the start and end of the test.

The protocol was reviewed and approved by the University of Wisconsin—Madison College of Agriculture and Life Sciences Animal Care Committee. The study was carried out under the supervision of Z.A. Din (DVM, M.Sc.), and S.G. Yu, (MD, PhD). The study was carried out under FDA-approved IND number 36906.

Assays of serum lipid parameters. The analyses of the coded samples were performed as described in detail recently (29) at Advanced Medical Research (Madison, WI). The serum total cholesterol, HDL cholesterol, and triglyceride concentrations were estimated by using kits 352 and 336, respectively, from Sigma Chemical Co. (St. Louis, MO). Serum LDL cholesterol was precipitated from 200 μL of serum with 25 μL of a mixture of 9.7 mM phosphotungstic acid and 0.4 M $MgCl_2$. The preparation was mixed for 10 min at room temperature and then was centrifuged at 12,000 × *g* for 20 min. The supernatant was decanted and analyzed for HDL cholesterol. The remaining precipitate was dissolved in 200 μL of 0.1 M sodium citrate, and the concentration of LDL cholesterol was estimated as described for total cholesterol (29). All assays for each treatment were carried out at the same time under similar conditions to minimize standard deviation.

Statistical analysis. The overall analyses identified significant main effects and interactions for all serum lipid parameters between the groups evaluated. StatView software (version 4.01, Abacus Concepts, Berkeley, CA) was used for the analyses of treatment-mediated effects as compared with respective control groups. Treatment-mediated differences in serum lipid (total cholesterol and LDL cholesterol) variables were identified with a two-way ANOVA, and when the F test indicated a significant effect, the differences between the means were analyzed by a Fisher's protected least significant difference test. Data were reported as mean ± SD in the text and tables. The statistical significance level was set at 5% ($P < 0.05$). The reductions achieved with the supplements at specified dose levels were also evaluated.

RESULTS

Effects of TRF, α-tocopherol, and α-, γ-, and δ-tocotrienols on serum lipid parameters in 5-wk-old female chickens. The previously reported cholesterol-suppressive action of TRF (19–21) is confirmed by plots of the data, which show the significant dose-dependent impact of TRF on serum total cholesterol (Fig. 1) and LDL cholesterol levels (Fig. 2). The maximum lowering of both parameters, a 22% reduction in total cholesterol and a 50% ($P < 0.05$) lowering of LDL cholesterol, was achieved with 1,000 ppm TRF. Increasing the α-tocopherol content of the diet by 5- to 200-fold (50-2,000 ppm) had no impact on serum total cholesterol level (Fig. 1) and yielded a modest lowering of LDL cholesterol (6%) only when present at supranormal (2,000 ppm) levels (Fig. 2).

Consistent with findings that the tocotrienol isomers have differential effects on HMG CoA reductase activity (25,29), α-tocotrienol was less effective than either γ-tocotrienol or δ-tocotrienol in lowering serum total (Fig. 3) and LDL (Fig. 4) cholesterol levels in the present study. A 500 ppm supplement of α-tocotrienol yielded the maximum response, a 17% reduction, whereas significant dose-dependent impacts of γ-tocotrienol and δ-tocotrienol were achieved ($P < 0.05$); for these isomers, 2,000 ppm supplements effected modest but significantly greater reductions than those attained with1,000 ppm supplements (Fig. 3). As shown in Figures 3 and 4, 500 ppm/kg diet supplementation of α-tocotrienol, γ-tocotrienol, and δ-tocotrienol effected 17%, 20%, and 27% ($P < 0.05$) reductions in serum total cholesterol levels, respectively. The serum total cholesterol level was decreased with TRF, 17% at 500 ppm/kg diet, which falls close to the average value of 17% recorded for its individual tocols: α-tocopherol (3%), α-tocotrienol (17%), γ-tocotrienol (20%), and δ-tocotrienol (27%), at 500 ppm/kg diet of each supplement (Figs. 1 and 2).

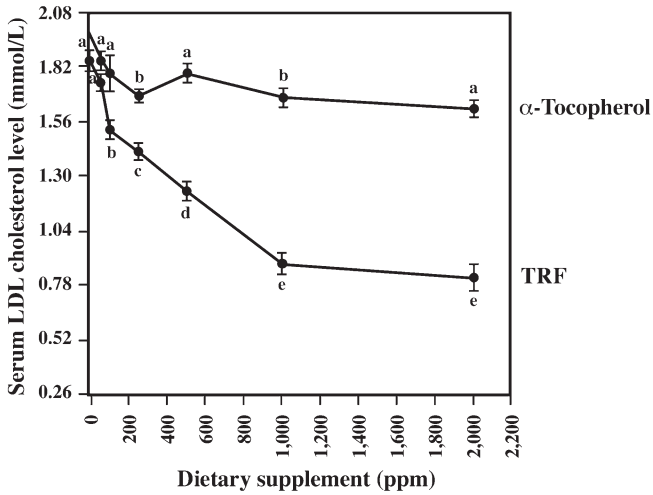

FIG. 2. Dose-dependent impacts of the TRF from palm oil and α-tocopherol on the serum LDL cholesterol levels of pullets. Values are means ($n = 6$); standard error is shown by bars. Points on a line not sharing a common letter are significantly different ($P < 0.05$).

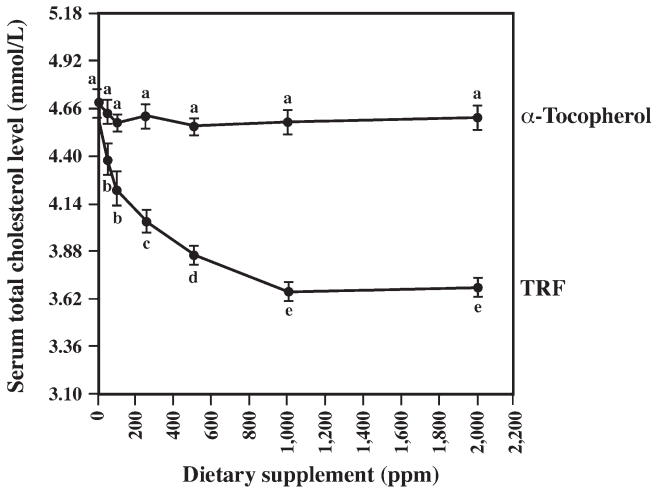

FIG. 1. Dose-dependent impacts of the TRF from palm oil and α-tocopherol on the serum cholesterol levels of pullets. Values are means ($n = 6$); standard error is shown by bars. Points on a line not sharing a common letter are significantly different ($P < 0.05$).

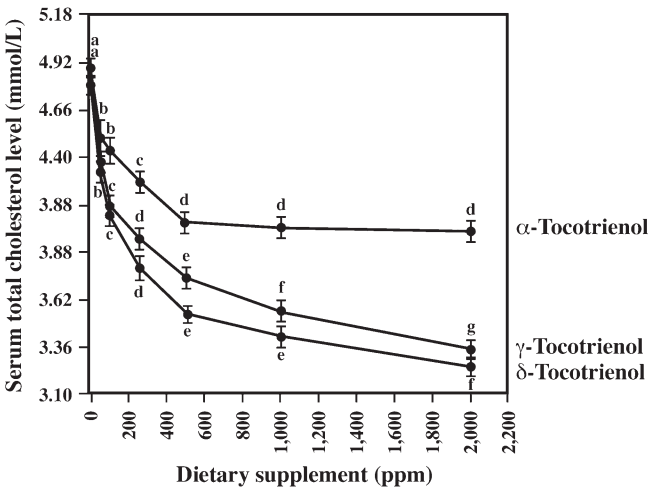

FIG. 3. Dose-dependent impacts of the tocotrienol isomers on the on the serum cholesterol levels of pullets. Values are means ($n = 6$); standard error is shown by bars. Points on a line not sharing a common letter are significantly different ($P < 0.05$).

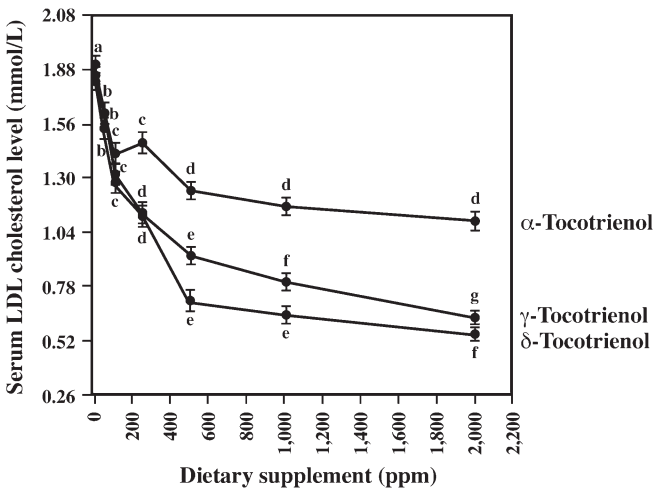

FIG. 4. Dose-dependent impacts of the tocotrienol isomers on the serum LDL cholesterol levels of pullets. Values are means ($n = 6$); standard error is shown by bars. Points on a line not sharing a common letter are significantly different ($P < 0.05$).

As noted in the experimental section, TRF consists of a blend of 16.8% α-tocopherol, 26.5% α-tocotrienol, 42.0% γ-tocotrienol, and 14.1% δ-tocotrienol. The 21% reduction in serum total cholesterol level obtained with a 2,000 ppm supplement of TRF is in agreement with the sum (22.1% reduction) of proportional impacts of the individual constituents.

The α-tocopherol supplement produced a significant, dose-dependent elevation of HDL cholesterol, confirming the finding reported by others (36–38); other supplements had less impact on HDL levels (Table 1). Although no supplement-mediated trends in triglyceride levels were observed, the levels in all supplemented groups receiving α-tocopherol, TRF, and δ-tocotrienol were significantly lower than the control value, as were the levels of the groups receiving the highest supplements of α- and γ-tocotrienols (Table 2). Weight gains were not impacted by the supplements; within each supplement grouping the weight gain by the group receiving the 2,000 ppm supplement matched that of the control. Similarly, differences in values for other parameters, such as feed consumption, body weight at sacrifice, and weights of liver, lungs, pancreas, kidneys, and heart, did not differ between treatments or within treatments (data not shown).

Figures 5 and 6 show the changes from control in total and LDL cholesterol levels produced by 500 ppm of each of the individual supplements. The various supplementation levels (50–1,000 ppm) of α-tocopherol did not reduce serum total (Fig. 5) or LDL (Fig. 6) cholesterol levels. On the other hand, 500 ppm supplementation of TRF or α-, γ-, or δ-tocotrienol caused significant lowering of serum total and LDL cholesterol

TABLE 1
Effects of Tocotrienol-Rich Fraction and Its Tocols on Serum HDL Cholesterol Levels in 5-Wk-Old Female Chickens[a]

	HDL Cholesterol Concentration in Serum (mmol/L)				
Concentration (ppm)	TRF[b]	α-Tocopherol	α-Tocotrienol	γ-Tocotrienol	δ-Tocotrienol
0	2.64 ± 0.02^{a}	2.64 ± 0.02^{a}	2.72 ± 0.02^{b}	2.63 ± 0.02^{a}	2.69 ± 0.03^{bc}
50	2.64 ± 0.03^{a}	2.63 ± 0.03^{a}	2.74 ± 0.03^{bc}	2.72 ± 0.02^{b}	2.66 ± 0.03^{ab}
100	2.66 ± 0.02^{a}	2.74 ± 0.01^{b}	2.73 ± 0.02^{bc}	2.71 ± 0.02^{b}	2.65 ± 0.02^{ab}
250	2.63 ± 0.02^{a}	2.76 ± 0.02^{b}	2.63 ± 0.03^{a}	2.70 ± 0.01^{b}	2.64 ± 0.02^{ab}
500	2.60 ± 0.03^{a}	2.79 ± 0.02^{bc}	2.76 ± 0.02^{bc}	2.72 ± 0.01^{b}	2.68 ± 0.01^{bc}
1,000	2.59 ± 0.03^{a}	2.77 ± 0.03^{bc}	2.78 ± 0.02^{bc}	2.73 ± 0.01^{b}	2.63 ± 0.02^{ab}
2,000	2.60 ± 0.02^{a}	2.81 ± 0.01^{c}	2.79 ± 0.02^{c}	2.70 ± 0.01^{ab}	2.62 ± 0.01^{a}

[a]Feeding period was 4 wk. Time of collecting the blood was 08:00 h. The chickens were fasted for 12 h prior collecting the blood samples. Data are expressed as means ± SD; $n = 6$ chickens per group.
[b]TRF, tocotrienol-rich fraction from palm oil (α-tocopherol, 16.8% + α-tocotrienol, 26.5% + γ-tocotrienol, 42.0% + δ-tocotrienol, 14.1%).
[a–c]Values in the same column not sharing a common superscript letter are significantly different at $P < 0.05$.

TABLE 2
Effects of TRF and Its Tocols on Serum Triglyceride Levels in 5-Wk-Old Female Chickens[a]

	Triglyceride Concentration in Serum (mmol/L)				
Concentration (ppm)	TRF[b]	α-Tocopherol	α-Tocotrienol	γ-Tocotrienol	δ-Tocotrienol
0	2.14 ± 0.014^{a}	2.15 ± 0.014^{a}	2.06 ± 0.010^{a}	1.96 ± 0.010^{abc}	2.07 ± 0.010^{a}
50	2.07 ± 0.015^{bc}	2.03 ± 0.011^{b}	2.04 ± 0.010^{ab}	1.97 ± 0.010^{a}	1.92 ± 0.010^{c}
100	2.09 ± 0.014^{b}	2.01 ± 0.010^{bcde}	2.02 ± 0.013^{ab}	1.97 ± 0.010^{ab}	1.93 ± 0.011^{bc}
250	2.04 ± 0.013^{cde}	2.02 ± 0.011^{bcd}	2.02 ± 0.013^{ab}	1.94 ± 0.013^{abcd}	1.95 ± 0.010^{b}
500	2.07 ± 0.011^{cde}	1.98 ± 0.018^{e}	2.01 ± 0.011^{ab}	1.95 ± 0.010^{abcd}	2.01 ± 0.011^{c}
1,000	2.05 ± 0.011^{cde}	2.02 ± 0.011^{bc}	2.03 ± 0.031^{ab}	1.94 ± 0.012^{bcd}	1.92 ± 0.013^{c}
2,000	2.02 ± 0.010^{e}	2.01 ± 0.014^{bcde}	1.99 ± 0.012^{b}	1.93 ± 0.011^{d}	1.91 ± 0.012^{c}

[a]Feeding period was 4 wk. Time of collecting the blood was 08:00 h. The chickens were fasted for 12 h prior collecting the blood samples. Data are expressed as means ± SD; $n = 6$ chickens per group.
[b]TRF = tocotrienol-rich fraction from palm oil (α-tocopherol, 16.8% + α-tocotrienol, 26.5% + γ-tocotrienol, 42.0% + δ-tocotrienol, 14.1%).
[a–e]Values in the same column not sharing a common superscript letter are significantly different at $P < 0.05$.

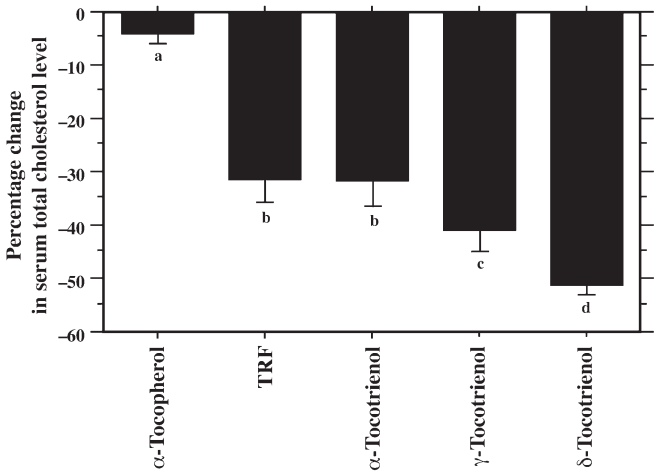

FIG. 5. Changes from control in serum total cholesterol levels of pullets receiving a 500 ppm supplement of TRF or its constituent tocols. Values are means ($n = 6$); standard error is shown by bars. Columns not sharing a common letter are significantly different ($P < 0.05$).

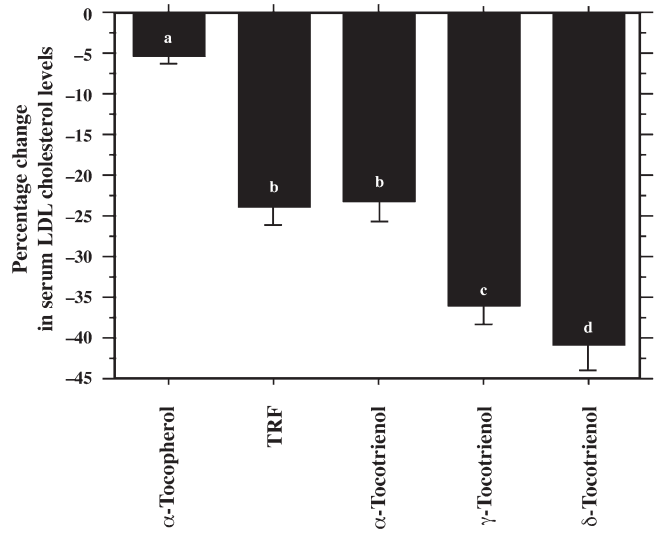

FIG. 6. Changes from control in serum LDL cholesterol levels of pullets receiving a 500 ppm supplement of TRF or its constituent tocols. Values are means ($n = 6$); standard error is shown by bars. Columns not sharing a common letter are significantly different ($P < 0.05$).

levels (Figs. 5 and 6). TRF and α-tocotrienol were effective in lowering these parameters; the reductions in the total and LDL cholesterol levels achieved with the γ-tocotrienol supplement were significantly greater than those achieved with the TRF and α-tocotrienol supplements, and they were significantly less than those achieved with the δ-tocotrienol supplement (Figs. 5 and 6). Supplementation with higher concentrations (1,000 ppm or 2,000 ppm) of these tocotrienols did not show the profound reductions in total and LDL cholesterol levels compared with control values that were found with 500-ppm supplementation.

DISCUSSION

The absence of a supplement-induced change in weight gain, weight of organs, or feed consumption suggests that the elevated levels of TRF and its constituent tocols were free of toxic effects. Consistent with widely published reports (19–22,24–29), the unsaturated tocol analogues and TRF supplements produced a significant dose-dependent lowering of total (Fig. 1) and LDL (Fig. 2) cholesterol levels. A lowering of LDL cholesterol was achieved with higher levels of α-tocopherol; 2,000 ppm showed a modest response compared with responses to TRF and the individual tocotrienols (Fig. 4), yielding a 6% reduction (Fig. 2). The relative impacts of the tocotrienol isomers on serum LDL cholesterol levels (Fig. 4) were parallel with but substantially greater than those recorded for total cholesterol levels (Fig. 3). The 55% reduction in LDL cholesterol level obtained with a 2,000-ppm supplement of TRF is modestly greater than the sum, a 50% reduction in the LDL cholesterol level, of the proportional impacts of the individual constituents.

These results show that the serum total and LDL cholesterol levels were decreased significantly ($P < 0.05$) in a dose-dependent (50–2,000 ppm) manner by individual tocotrienols (Figs. 1–4). These decreases in cholesterol levels induced by tocotrienols were due to down-regulation of HMG-CoA reductase activity by a novel post-transcriptional mechanism, which results in decreased protein synthesis of HMG-CoA reductase, while increasing the degradation of the reductase protein (22). Therefore, cholesterol synthesis *in vivo* was suppressed, and concomitantly, the levels of total and LDL cholesterol were decreased. The pure individual tocotrienols were more effective in lowering the serum total and LDL cholesterol levels as compared with TRF, which typically contains 16% to 45% α-tocopherol in various preparations of palm oil or rice bran oil (39,40). As pointed out earlier, tocotrienols act by inhibiting the enzymic activity of HMG-CoA reductase; however, α-tocopherol induces HMG-CoA reductase activity in the liver without affecting serum cholesterol levels (39,40).

The α-tocopherol supplements, in contrast with the TRF and tocotrienol supplements, tended to raise HDL cholesterol levels. Nevertheless, the HDL/LDL ratio remained constant across the dose range for the α-tocopherol supplements, whereas the ratio improved dramatically in a dose-response relationship for TRF and tocotrienol supplements (Fig. 7). Therefore, the individual tocotrienols not only lower LDL cholesterol, but also improve the ratios of HDL cholesterol to total cholesterol and LDL cholesterol significantly, 200–300% (Fig. 7).

The present findings indicate that supplementation of 500 ppm of α-tocotrienol, γ-tocotrienol, and δ-tocotrienol will yield a reduction of 0.47, 0.62, and 0.74 mmol/L, respectively, in serum total cholesterol levels. The supplementation at the 50 ppm level caused decreases of 0.36, 0.44, and 0.57 mmol/L (Fig. 3). This differential impact of the individual tocotrienols on serum total cholesterol levels parallels their differential impacts *in vitro* on hepatic HMG-CoA reductase activity (25,29).

Tocotrienols show significant lowering of serum total cholesterol level in all animal models (19,22,24–29,31,32,39,40). On the other hand, human studies have yielded conflicting results; some studies indicate significant lowering of cholesterol

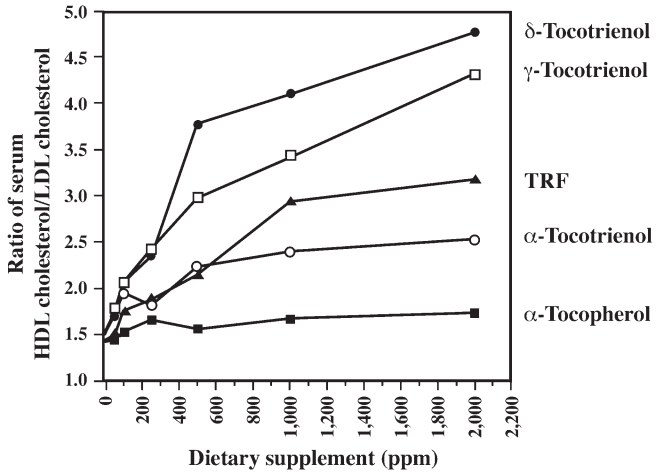

FIG. 7. Dose-dependent impacts of the TRF from palm oil and α-tocopherol on the HDL/LDL ratios of pullets.

levels (20,41–44), whereas in other studies, no response was detected (45–49). The differences observed in the human studies were partly due to the use of various doses and sources of TRF and tocotrienols. In order to compare the results of animal and human studies, Raederstorff *et al.* studied steady-state plasma γ-tocotrienol levels and plasma cholesterol levels in hamsters (50). They observed that the maximum effect of γ-tocotrienol was reached after 2 wk with the highest dose (263 mg/d/kg body weight); thereafter, the effect on cholesterol was plateaued. However, the cholesterol level continued to decrease after week 2 with the intermediate dose (58 mg/d/kg body weight). The lowest dose (23 mg/d/kg body weight) did not lower total cholesterol levels in their study, as was also reported by Hayes *et al.* (51), due to the presence of very low plasma level of tocotrienols (1 μmol/L). The only significant decrease in plasma cholesterol levels, in the hamster model, was seen when plasma tocotrienols levels were above 15 μmol/L with a dose of 58 mg/kg body weight (50).

The plasma levels of tocotrienols were 5–8 μmol/L (200 mg/d) in our first human study (20). On the other hand, the plasma levels of tocotrienols were 0.2 μmol/L and 1 μmol/L in the Mensik *et al.* (47) and O'Byrne *et al.* (48) studies, as pointed out by Raederstorff *et al.* (50). Therefore, it seems that in several well-designed human studies, pharmacologically effective plasma levels of tocotrienol were not reached, which could explain the inconsistent effects observed in various human studies (45–49), including some nonrespondents in our studies (20,42–44). The second possible explanation of reported inability of tocotrienols to lower serum total and LDL cholesterol levels in hypercholesterolemic human subjects is the presence of higher percentages of tocopherols (>30%) in the TRF used by various investigators (45–49).

As mentioned earlier, α-tocopherol appears to attenuate the tocotrienol effect *in vivo* but not in isolated liver cells (25). These findings suggest that transport systems may play a role in the biological activities of tocols. The differences in the transport and tissue uptake of the saturated (α-, γ-, and δ-tocopherols) and unsaturated (α-, γ-, and δ-tocotrienols) tocols have been reported by various investigators (51–54). The preferential transport of α-tocopherol by α-tocopherol transfer protein (α-TTP) in serum lipoproteins was reported by Traber *et al.* (52). A hepatic binding protein with high specificity for α-tocopherol in the liver results in the subsequent enrichment of α-tocopherol in the VLDL and LDL moieties (52–54). The tocotrienols, on the other hand, are transported nonspecifically like other lipid-soluble compounds, and are found in much higher levels in the HDL moiety (42). α-Tocotrienol has preferential absorption compared with γ- or δ-tocotrienol in rat LDL moiety due to low affinity by hepatic α-TTP (55), and these tocotrienols also might be competing with α-tocopherol in the TRF supplements used by others in their studies (56). Moreover, these various tocotrienol isomers may have been retained in tissues to different extents (56). The tocotrienols cleared rapidly from plasma due to low affinity of tocotrienols to hepatic α-TTP, which may also account for the ambiguous findings by various investigators (56). In this respect, a recently published human study investigating the pharmacokinetics of a 300-mg dose is of relevance (57). The pharmacokinetics and bioavailability of various tocotrienols in postprandial and fasting humans were reported (57). The absorption of each tocotrienol was 100% greater in the fed state, and their elimination half-life ($t_{1/2}$) was found to be relatively short compared with tocopherol. The plasma levels of all tocotrienols were markedly increased in the fed state and were achieved between 3 and 5 h. The experimental values of various tocotrienols compared with the pharmacokinetic results of α-tocopherol were described in detail (57).

Thus, in chickens, palm oil preparations containing at least 30% α-tocopherol and 45% γ-tocotrienol seem to be less effective in lowering cholesterol than preparations containing 15–20% α-tocopherol and 60% γ-tocotrienol (39). Similarly, lower bioavailability of tocotrienyl acetates compared with the phenolic forms may have contributed to the lack of effect in the O'Byrne *et al.* human study (48). It should be noted that only clinical trials with duration of less than 35 d have provided evidence that γ-tocotrienol or mixed tocotrienols/tocopherol preparations lower serum total and LDL cholesterol levels, suggesting that tocotrienols have only short-term inhibitory effects on cholesterol synthesis (42–44).

The third possible reason of the inability of tocotrienols to lower serum total and LDL cholesterol levels in hypercholesterolemic human subjects might be due to the presence of higher percentages of tocopherols (>30%) in TRF used by various investigators (45–47,49). Khor *et al.* (58) have reported that a tocopherol-free TRF preparation caused a dose-dependent increase in the α-tocopherol content of guinea pig liver and serum, which confirmed our earlier findings that dietary TRF or γ-tocotrienol causes an increase in serum α-tocopherol levels in hypercholesterolemic humans (42–44). The failure of large doses of tocotrienols as effective hypocholesterolemic agents in all of these studies might be due to their

conversion to α-tocopherol *in vivo*, because the level of α-tocopherol in serum of TRF- or pure tocotrienol–treated subjects is substantially higher (2- to 4-fold) compared with the placebo group, as reported also by other investigators (20,45–47,49,50,58). This bioconversion of γ-tocotrienol to α-tocopherol was confirmed by feeding radioactive γ-[4-^{3}H]-tocotrienol to chickens for 4 wk, followed by the separation of various tocols from serum by HPLC. The radioactivity was found only in α-tocopherol, α-tocotrienol, and γ-tocotrienol, but not in δ-tocopherol or δ-tocotrienol (43). These findings were further supported by other investigators (27,37,50,58).

The recent studies show that CRP (associated with inflammation) is playing a more important role in the development of atherosclerosis than the previously established lipid parameters, total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides (4,5). Preliminary data in an ongoing open human study (5 hypercholesterolemic subjects) shows a decrease (11%) in CRP after feeding δ-tocotrienol (100 mg/d) for 4 wk (unpublished results). Atherosclerosis is a disease of chronic inflammation and accumulation of cholesterol and foam cells within the arterial wall (1–3). Our recent studies indicate that tocotrienols reduce the induction of tumor necrosis factor-α (TNF-α) in response to lipopolysaccharide (Re-LPS) challenge in mice and macrophages (59). δ-Tocotrienol also blocks LPS-induced gene expression of TNF-α, IL-β, IL-6, and iNOS in thioglycolateelicited peritoneal macrophages prepared from BALB/c, C57BL/6N, and PPAR-α mice (data not shown). Tocotrienols also prevent atherosclerotic lesions in apo-E–deficient mice and rabbits due to reduction of inflammation (32,60).

Tocotrienols in various human studies (consuming doses of 50–200 mg/d) were not very effective for lowering serum total cholesterol levels in all hypercholesterolemic subjects, with 20–25% nonrespondent (20,42–44). Therefore, a higher oral dose of tocotrienols might be required to reach an effective level in plasma to suppress cholesterol synthesis. A high level of tocotrienols or α-tocopherol (200–1,000 mg/d) could safely be consumed by humans on the basis of the present study; however, one should exercise care in extrapolating the studied high doses of tocotrienols for human consumption.

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Influence of Diet on Fatty Acids and Tocopherols in *M. Longissimus Dorsi* from Reindeer

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ABSTRACT: Our aim was to compare the effects of two pelleted diets containing differing FA composition with natural lichen pasture on reindeer's meat FA composition. In addition we wanted to increase the knowledge about reindeer FA metabolism and the effect of animal sex and age on FA composition in reindeer muscle.

The trial included five reindeer groups: three grazing, consisting of adult males, adult females, and calves; and two groups of calves fed conventional pellets (CPD) and pellets containing crushed linseed (LPD), respectively, for two months before slaughter. Differences between male and female animals were mainly found in the neutral lipid fraction and related to fatness. Calves differed significantly from adult males and females in FA and lipid class composition. CPD led to a higher ratio of n-6 to n-3 FA compared with grazing. The ratio n-6/n-3 in the polar lipid (PL) fraction of the animals fed LPD was slightly, but not significantly, higher than that in the grazing reindeer. LPD-fed animals had lower proportions of long-chain polyunsaturated FA (LCPUFA), namely 20:4n-6, 22:5n-3, and 22:6n-3, in the PL fraction compared with the grazing animals due to the content of these FA in the natural feed. The animals seemed unable to elongate dietary FA in significant amounts. We conclude that by adding crushed linseed to the pellets it was possible to keep the favorable FA composition of meat from grazing reindeer with regard to the n-6/n-3 ratio but not in LCPUFA.

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A diet rich in polyunsaturated FA (PUFA), especially n-3 FA, has beneficial effects on human health (1). Today's Western diets have an n-6/n-3 ratio between 15 and 20, whereas it is assumed that it was close to 1 during human evolution (2,3). Because from a human nutritional point of view it is suggested that the n-6/n-3 ratio in the diet should be below 4, it is important to include sources rich in n-3 PUFA in the daily diet. There is an interest and a need to improve FA composition of meat and to produce healthier meat (4). The FA composition of the animal's diet influences the FA composition of the meat (4). Meat from grazing reindeer has been suggested to be nutritionally valuable because of its low fat content and moderate proportion of n-3 PUFA (5).

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Abbreviations: CPD, conventional pellets; GLM, general linear model; HIP, hexan-isopropanol; LCPUFA, long-chain polyunsaturated FA; LPD, linseed pellets; MUFA monounsaturated FA; NL, neutral lipid fraction; PL, polar lipid fraction; SFA, saturated FA; SPE, solid phase extraction.

Reindeer are among the northernmost freely ranging ruminants in Scandinavia, and are well adapted to their habitat, which has a cold climate and snow during the main part of the year. In Sweden all reindeer are semidomesticated, and reindeer husbandry is based on utilization of natural pastures all year round. The reindeer migrate from pastures on the mountain tundra in the summer months to winter pastures in the valleys and forests, so their diet changes markedly over the year (6). The main diet for reindeer during winter before slaughter consists normally of wintergreen plants and lichens (7). However, occasionally, an increased number of reindeer are fed commercial pellets before slaughter, during winter. The main reason to feed pellets is to reduce radioactive cesium (Cs) in the meat of reindeer that have been winter grazing in certain contaminated areas (8). Partly, feeding is also used to improve the nutritional status (9) and enable weight gain of the animals, because reindeer normally do not gain weight at winter pastures (10).

Taugbøl and Mathiesen (11) found changes in the FA composition of total lipids of reindeer meat due to variation in feed composition, and Wiklund *et al.* (5) showed a difference in the FA profile in neutral lipids (NL) and polar lipids (PL) between different feeding groups. FA composition of ruminant tissues is in general less influenced by the feed than that of monogastric animals (12). In ruminants a high proportion of C18 unsaturated FA are hydrogenated in rumen by microorganisms if they are not naturally protected by cell walls as in plants or by synthetic coatings (13). Because of this hydrogenation, feeding of concentrates to ruminants usually leads to lower proportions of PUFA in the meat compared with monogastric animals fed on the same diet (12). This effect can be suppressed by adding whole or crushed seeds, instead of oils, to feeds (13,14). In addition, intensive feeding with pellets leads to a higher n-6/n-3 ratio in the meat because concentrates are often based on grains that are rich in n-6 FA, whereas grass and other green plants are rich in n-3 FA (14). It has also been suggested that a higher intake of 18:3n-3 could enhance *de novo* synthesis of longer-chain n-3 PUFA, such as 20:5n-3 and 22:5n-3 (15) (cattle).

The purpose of this study was to investigate the possibility of improving the FA composition of the meat from pellet-fed reindeer. To achieve a more nutritionally favorable composition in the pellets, linseed, a rich source of 18:3n-3, was added, mimicking the FA composition of natural green forage in terms of n-6/n-3 ratio. Meat lipids were separated into

NL and PL fractions because a variation of total lipid content will lead to different proportions of these fractions. NL represents the storage lipids, which are mainly used as a depot for energy sources, whereas PL refers to the membrane lipids, which represent a relatively stable amount (15). Studies on different species exemplify that age and sex might influence lipid metabolism and thereby FA composition in the muscle (16–19) (goats, pigs, and cattle). We therefore chose to include animals of different age and sex in this study. However, because earlier results showed that FA composition of meat did not differ between male and female reindeer calves (20), calves were not separated by sex.

In addition, we studied how diet, age, and sex affected tocopherol and retinol content in the meat, because tocopherol is an important antioxidant preventing oxidation of FA.

EXPERIMENTAL PROCEDURES

Animals. A total of 38 reindeer (7 males and 7 females aged 2 yr and older, and 24 calves aged about 10 mon) were included in the study. Age was determined at slaughter by body size and the color of the knee conjunction. Animals 2 yr and older were considered as adult animals. The older animals and a group of 7 calves were previously grazing winter pasture, whereas the other calves were divided in two groups ($n = 10$ and $n = 7$, respectively) that were fed two different pelleted feed mixtures as complete feeds *ad libitum*. One of these latter groups was fed a conventional pellet diet (CPD), and the other was fed pellets enriched with 6% crushed linseed (linseed pellet diet, LPD) (Table 1) (21) for two months before slaughter. In addition a small amount of hay was offered as roughage. All animals were slaughtered in early April, following the common procedures at the abattoirs (Grundnäs Kött AB, Grundnäs and Arvidsjaur Renslakt AB, Arvidsjaur, Sweden). All animals were in good condition; slaughter weights and classification were presented previously in the literature (22). Fresh meat samples were taken directly after slaughter (about 45 min *post mortem*) from the *M. longissimus dorsi*. All samples were frozen at –20°C and finally stored at –80°C until analysis.

TABLE 1
Industrial Nutrient Declaration for Renfor Bas and Renfor Bas Enriched with Crushed Linseed (21)

Nutrient content	Renfor Bas (CPD)	Renfor Bas plus 6% linseed (LPD)
Crude protein, %	10.0	10.4
Energy, MJ/kg	10.0	10.0
Fiber, %	15.0	14.6
Crude fat, %	3.0	3.05
Water, %	12.0	12.0
Vitamin A, IU/kg	9,000 (2.7 mg/kg)	9,000 (2.7 mg/kg)
Vitamin D_3, IU/kg	3,000 (0.075 mg/kg)	3,000 (0.075 mg/kg)
Vitamin E, mg/kg	60	60
Calcium, %	0.8	0.8
Phosphate, %	0.4	0.4
Magnesium, %	0.3	0.3
Selenium, %	0.6	0.6

Feed samples. Samples of the pellets and different lichens (*Cladina arbuscula*, *Cetraria islandica*, *Cladina stellaris*, and *Cladina mitis*) from the grazing area were collected. The dry lichens were stored at –80°C until analyzed.

Extraction of lipid from the meat. The lipid extraction was performed according to Hara and Radin (23), with slight modifications as described by Pickova *et al.* (24). Connective tissue and visible fat were removed, the semifrozen samples were minced, and a subsample of approximately 5 g muscle tissue was taken for extraction. The samples were homogenized for 3 × 30 s in 70 mL hexane/isopropanol (HIP) (3:2, vol/vol) using an Ultra Turrax (T25, Janke and Kunkel, IKA Werke, Germany), and 30 mL Na_2SO_4 solution (0.47 M) was added. The homogenate was centrifuged for 5 min at 4000 rpm (2103 rcf) (Sorvall Super T21; Sorvall Products L.P., Newton, CT) and the upper phase was transferred to a new flask and evaporated under nitrogen. The lipid content of the meat was determined gravimetrically from this total extracted lipid, which was then dissolved in 2 mL chloroform. The samples were stored at –80°C in normal atmosphere until further analyses.

Extraction of lipid from the feed. The different feed samples were extracted according to Folch *et al.* (25), with slight modifications. Lichens and pellets were milled, and subsamples of 2 g dry material were soaked in 8 mL H_2O. The samples were then homogenized for 3 × 30 s in 150 mL chloroform/methanol (2:1, vol/vol) using an Ultra Turrax (T25, Janke and Kunkel). The samples were transferred to a separatory funnel and 40 mL KCl solution (0.11 M) was added. The lower phase, containing the lipids, was transferred to a new flask and evaporated. The lipid content from the lichens and the pellets was determined from this total extracted lipid, which was then dissolved in 2 mL chloroform. The samples were stored at –80°C in normal atmosphere until further analyses.

Separation of the meat lipids. Total lipids from the meat were separated on prepacked 6-mL solid-phase-extraction (SPE) columns (Isolute SI 500 mg, IST, United Kingdom) into NL and PL fractions according to Prieto *et al.* (26) with slight modifications. The samples were dissolved in 2 mL diethylether/acetic acid (100:0.2, vol/vol) and applied on the columns, which were activated previously with 6 mL hexane. NL were eluted with 18 mL hexane/diethylether (200:3, vol/vol) and the PL fraction with 6 mL methanol/acetonitrile (65:35, vol/vol). After evaporating the solvent, the lipids were dissolved in 0.5 mL hexane and stored under normal atmospheric pressure at –80°C until further analysis.

Preparation of FAME of PL and NL. FA from the NL and PL fractions were methylated according to Appelqvist (27). To each sample, 2 mL of a 0.01 M solution of NaOH in dry methanol was added, after which the samples were heated for 30 min at 60°C. Thereafter 2 mL of a solution of $NaHSO_4$ and NaCl in water (4.16 and 4.27 M, respectively) was added. After cooling the samples under running water, 3 mL water was added and the FAME were extracted with 2 mL hexane, transferred to a new tube, evaporated, dissolved again, and

stored under normal atmosphere at –20°C for a maximum of 1 wk until analysis.

Preparation of FAME of total lipids from feed samples. FA from total lipids of lichens and pellets were methylated according to Appelqvist (27). To each sample, 2 mL of a 0.01 M solution of NaOH in dry methanol was added, and the samples were then heated for 10 min at 60°C. Next, 3 mL of BF_3 reagent (boron trifluoride-methanol complex) was added and the samples were reheated at 60°C for 10 min. Thereafter the tubes were cooled under running cold water and 2 mL of a 3.42 M NaCl solution in water was added to all tubes. The FAME were extracted with 2 mL hexane, and the upper layer was transferred to a new tube and evaporated under nitrogen gas to dryness. The lipids were dissolved in 0.5 mL hexane and stored under normal atmospheric pressure at –20°C for a maximum of 1 wk until GC analysis.

Capillary GC. The completeness of FA methylation was checked by analytical TLC using hexane/diethyl ether/acetic acid (85:15:1, by vol) as solvent. The plates were developed by spraying with a mixture of 10% phosphomolybdic acid in ethanol:diethylether (1:1, vol/vol) and drying for 15 min at 100°C. The FAME were analyzed with a gas chromatograph (CP9001, Chrompack, Middelburg, The Netherlands) equipped with an FID and split injector as described by Pickova *et al.* (24) using a BPX 70 column (SGE, Austin, TX) with length 50 m, i.d. 0.22 mm, and film thickness 0.25 μm. The GC was programmed to start at 158°C, with a rate of temperature increase of 2°C/min to 220°C and a final constant time of 13 min at 220°C. Helium was used as carrier gas at a flow rate of 0.8 mL/min. Makeup gas was nitrogen. The peaks were identified by comparing their retention times with those of the standard mixture GLC-68A (Nu-Chek-Prep, Elysian, MN) and other authentic standards. The response factors were also evaluated by comparing with the GLC-68A standard. An average of 95.6% of the peak areas could be identified. The percentages of FA were calculated as 100% from the FA identified. The unknown peaks in both NL and PL fractions, including the dimethyl acetals from the phospholipid plasmalogens (28), were subtracted before addition due to the high SD, which otherwise might have disturbed the calculation of the percentage of the other FA. The proportion of excluded unknowns was on average 10.2% of the identified FA in PL fraction and 2.2% of the identified FA in NL fraction.

Vitamin analysis. For the analysis of retinol and tocopherols in meat, pellets, and lichens a method described by Jensen *et al.* (29), slightly modified, was used as follows: 2 × 1 g muscle (0.5 g for pellets and lichens) was ground and homogenized in two tubes together with 1.2 mL 20% ascorbic acid solution, 0.6 mL methanol, and 1.2 mL 17.8 M KOH. After saponification and cooling, tocopherols and retinol were extracted with 2 × 4 mL hexane. The hexane-vitamin solution was evaporated under nitrogen gas and diluted with the mobile phase. The mobile phase used consisted of 95% methanol:acetonitrile (1:1, vol/vol) and 5% chloroform pumped at a flow rate of 1.2 mL/min. Analyses were carried out with a Merck Hitachi L7100 pump, an FL-7485 detector, and an L-7200 auto sampler (Merck Hitachi, Eurolab, Darmstadt, Germany). The HPLC column was a 4.0 × 250 mm RP-18 LiChroCART (Merck KGaA, Darmstadt, Germany).

Identification and quantification were done by external standards. Retinol was detected with an excitation wavelength of 344 nm and emission wavelength of 472 nm; γ-tocopherol and α-tocopherol were detected with excitation wavelengths of 290 and 260 nm, respectively, and with an emission wavelength of 327 nm.

Statistical analysis. The statistical analyses were carried out with the Statistical Analysis System (30) using the general linear model (GLM) procedure. The models for comparing fat content, FA, and vitamins included age, gender, and the treatment of the animals as fixed factors.

RESULTS

*Composition of the feed***.** The content of retinol and tocopherols was higher in the pellets than in the lichens. The content of tocopherol acetate in the CPD was slightly higher than in the LPD (Table 2).

The pelleted feed contained more saturated FA (SFA), especially 12:0, compared to the lichens, due to the use of palm-expeller in the pellets (Table 2). The n-6/n-3 ratio in the lichens was around 5.3–5.8 except for *Cetraria islandica*, which had a lower ratio of 2.7. The pelleted feed had an n-6/n-3 ratio of 4.3 for the CPD and 2.6 for the LPD.

Fat content of different lichens was lower than the fat content of the pellets except for *Cetraria islandica*, which had a higher fat content than the pellets.

Fat content and FA composition of the meat. The adult females had the highest fat contents, followed by the males, whereas the pellet-fed calves had a slightly but not significantly higher fat content compared with the grazing calves, which had the lowest fat content (Tables 3 and 4).

Effect of age and sex on meat FA composition. FA composition in both PL and NL fractions differed only slightly between grazing calves and the grazing adult animals (Table 3, Fig. 1). Calves had a significantly lower proportion of 16:0 in both PL and NL and significantly higher proportions of 18:1n-9 and monounsaturated FA (MUFA) in the NL fraction than the adult animals. In the PL the proportions of SFA and MUFA were lowest in the calves, whereas n-3 PUFA was higher in these samples, compared with the adult animals. In the NL fraction SFA was significantly lower in the meat from the calves, whereas n-3 and n-6 PUFA were significantly higher compared with the adult animals. The n-6/n-3 ratio was significantly lower in the NL fraction of the calves compared with the males. No 22:6n-3 was found in NL fractions.

Effect of the different diets on meat FA composition. In spite of the high content of 12:0 in the pelleted feed, this FA is not detectable in significant amounts in either lipid fraction (Table 4).

The main differences in FA composition were found in the PL fraction of meat from animals fed different diets (Table 4, Fig. 2). In the meat from reindeer fed LPD the proportion of

TABLE 2
Fat Content, Retinol, γ- and α-Tocopherol, and FA Composition of Pellets and Various Lichens (% of total identified FA)[a]

	Conventional pellets (CPD)[b]	Linseed pellets (LPD)[b]	*Cladina mitis*	*Cladina stellaris*	*Cretaria islandica*	*Cladina arbuscula*
Fat content, %	3.75	3.93	2.39	2.31	4.67	3.16
Retinol, μg/g	4.00	2.08	0.81	0.93	0.44	0.55
γ-Tocopherol, μg/g	2.44	3.04	n.d.	0.04	n.d.	n.d.
α-Tocopherol, μg/g[b]	56.5	48.4	9.38	15.8	15.7	10.7
12:0	7.05	6.08	0.64	0.14	0.47	0.55
14:0	2.75	2.42	0.69	0.28	0.66	0.58
Unknown	0.04	0.03	0.27	0.23	0.37	0.27
16:0	17.4	19.3	9.51	8.62	9.38	9.19
17:0	trace	n.d.	0.23	0.19	0.37	0.14
18:0	2.27	2.59	4.66	3.19	2.83	4.13
20:0	0.30	0.24	0.92	0.31	0.47	1.20
22:0	0.28	0.20	1.70	0.50	0.93	2.48
24:0	0.24	0.23	1.17	0.40	0.48	1.77
16:1n-7*trans*	0.06	0.06	0.48	0.41	0.74	0.56
16:1n-9	0.21	0.16	0.59	0.74	0.63	0.88
18:1n-9	21.8	20.1	22.3	29.2	30.5	29.7
18:1n-7	0.85	0.81	1.53	1.88	1.24	1.67
20:1n-9	0.57	0.44	0.25	0.27	0.23	0.22
18:2n-6	37.0	33.8	44.6	44.3	35.6	37.5
18:3n-6	trace	0.06	0.13	0.07	0.08	0.12
18:3n-3	8.75	13.00	8.01	6.18	12.5	5.70
20:2n-6	0.08	0.07	0.35	0.58	0.27	0.36
20:3n-6	n.d.	n.d.	0.07	0.05	0.07	0.30
20:3n-3	n.d.	n.d.	0.11	1.33	0.26	1.25
20:4n-6	n.d.	n.d.	0.81	0.52	0.77	0.67
20:5n-3	n.d.	n.d.	0.33	0.45	0.81	0.29
22:4n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:5n-3	n.d.	n.d.	0.12	trace	trace	0.19
22:6n-3	n.d.	n.d.	n.d.	n.d.	trace	trace
SFA	30.6	31.3	19.5	13.6	15.6	20.1
MUFA	23.7	21.9	25.5	32.6	33.5	33.2
n-6 PUFA	37.3	34.2	46.1	45.5	36.8	39.1
n-3 PUFA	8.75	13.0	8.57	7.97	13.7	7.47
n-6/n-3	4.26	2.64	5.38	5.78	2.70	5.26

[a]Values reported are means of duplicate values. FA are presented if at least one group had values ≥ 0.16%. In total 32 FA were identified. Abbreviations: n.d., not detected; SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA.
[b]Tocopherol acetate in pellets.

20:4n-6 was significantly lower compared with the grazing animals, while 18:2n-6 was higher. The meat from grazing animals contained more long-chain PUFA (LCPUFA, C20 and longer FA), both n-3 and n-6. Also significantly more 18:1n-9 was found in meat PL of this group, compared with both pellet-fed groups. The content of n-3 PUFA in muscle PL fraction of pellet-fed animals did not differ between CPD- and LPD-fed animals. However n-3 in the PL fraction from the CPD-fed group differed from that of the grazing animals, and LPD-fed animals had an intermediate value.

In NL the main FA, 16:0, 18:0, and 18:1n-9, did not differ between treatments. A lower proportion of n-3 was found in muscle of animals fed LPD than those grazing ($P = 0.010$). Even if the proportion of n-3 in NL did not differ between CPD- and LPD-fed reindeer in this study, the ratio of n-6/n-3 was significantly lower in the LPD-fed animals compared with the animals fed CPD. However the n-6/n-3 ratio in the meat of grazing animals was still lowest.

Vitamin content. The calves had significantly more α-tocopherol compared with the adult animals (Table 5) among the grazing animals, whereas retinol and γ-tocopherol were not affected by age or sex. There was more retinol and γ-tocopherol in the meat of the pellet-fed animals compared with the grazing animals (Table 6).

DISCUSSION

The analyzed composition of vitamins in the pellets (Table 2) corresponded well with the industrial nutrient declaration (Table 1). The amount of tocopherol acetate was slightly lower in the LPD compared with LPD, which suggests that LPD were more prone to oxidation due to the higher content of n-3 PUFA, and that some of the antioxidants were used during feed storage.

The fat content of the lichens was comparable to results in earlier studies; the values varied from 2.9 to 4.5% for differ-

TABLE 3
FA Composition of Free-Ranging Reindeer of Different Age and Sex (% of Total Identified FA)[a]

Polar lipids	Males (n = 7)	SD	Females (n = 7)	SD	Calves (n = 7)	SD	P
16:0	11.0[a]	0.36	11.6[a]	0.56	10.1[b]	0.72	<0.001
17:0	0.27	0.07	0.30	0.07	0.29	0.03	0.615
18:0	14.0	0.70	13.7	0.73	14.3	0.74	0.313
12:1	trace[a]		trace[a]		0.18[b]	0.03	<0.001
14:1	0.29[a]	0.08	0.24[a]	0.08	0.15[b]	0.02	0.002
16:1*trans*	0.40[a]	0.05	0.32[b]	0.09	0.32[b]	0.04	0.053
16:1n-9	2.00[a]	0.48	1.36[b]	0.27	1.33[b]	0.15	0.002
17:1	0.25[a]	0.08	0.17[b]	0.07	0.15[b]	0.04	0.027
18:1n-9	19.4	1.16	19.2	1.82	18.1	1.14	0.217
18:1n-7	1.38[a]	0.10	1.12[b]	0.10	1.44[a]	0.27	0.009
18:1n-5	0.12[a]	0.05	0.10[a]	0.07	0.21[b]	0.03	0.002
18:2n-6	22.4	1.90	22.1	1.38	22.6	1.01	0.839
18:3n-6	0.17	0.03	0.22	0.28	0.13	0.02	0.579
18:3n-3	1.47	0.27	1.50	0.30	1.25	0.22	0.187
20:2n-6	0.32	0.42	0.27	0.47	0.07	0.01	0.430
20:3n-6	0.94[a]	0.13	1.09[b]	0.15	1.05[a,b]	0.09	0.101
20:3n-3	0.30	0.03	0.31	0.04	0.32	0.06	0.738
20:4n-6	15.1	1.69	16.1	2.00	17.0	1.44	0.161
20:5n-3	2.72	0.50	2.86	0.49	3.22	0.54	0.195
22:4n-6	0.96	0.20	0.96	0.15	0.80	0.18	0.213
22:5n-3	5.54[a]	0.41	5.72[a,b]	0.28	6.03[b]	0.42	0.076
22:6n-3	0.47[a]	0.10	0.35[b]	0.09	0.51[a]	0.03	0.002
Neutral lipids							
14:0	1.91[a]	0.28	1.72[a,b]	0.23	1.59[b]	0.18	0.055
16:0	29.8[a]	1.89	28.7[a]	1.50	26.2[b]	2.02	0.005
17:0	0.88[a]	0.04	0.93[a,b]	0.07	0.97[b]	0.10	0.078
18:0	22.7[a]	1.74	26.4[b]	1.96	21.7[a]	1.58	<0.001
20:0	0.26	0.04	0.27	0.07	0.35	0.13	0.121
12:1	0.89[a]	0.27	0.56[b]	0.10	1.13[c]	0.14	<0.001
16:1*trans*	0.52[a]	0.10	0.41[a]	0.08	0.84[b]	0.21	<0.001
16:1n-9	1.92[a]	0.28	1.38[b]	0.19	1.56[b]	0.17	0.001
17:1	0.23	0.03	0.24	0.03	0.25	0.04	0.479
18:1*trans*	0.37[a]	0.04	0.39[a]	0.10	0.48[b]	0.06	0.027
18:1n-9	36.6[a]	1.90	35.4[a]	2.45	39.2[b]	1.91	0.009
18:1n-7	0.98[a]	0.12	0.65[b]	0.08	1.22[c]	0.25	<0.001
20:1n-9	0.22	0.06	0.22	0.05	0.28	0.07	0.096
18:2n-6	1.49[a]	0.17	1.54[a]	0.30	1.99[b]	0.46	0.023
18:3n-3	0.16[a]	0.06	0.25[b]	0.09	0.18[a,b]	0.04	0.060
20:4n-6	0.24[a]	0.06	0.21[a]	0.04	0.46[b]	0.20	0.003
22:5n-3	0.39[a]	0.23	0.38[a]	0.29	1.05[b]	0.78	0.033
Total lipids (%)	2.60[a]	0.69	4.17[b]	1.13	2.10[a]	0.31	<0.001

[a]Means with different superscripts within a row differ significantly ($P < 0.05$). FA are presented if at least one group had values > 0.15%. In total 32 FA were identified.

ent *Cladina* spp. from Finland (7) and were slightly lower in a Norwegian study (31). The FA composition of the lichens was different to that found by Soppela *et al.* (32); in particular, we found twice as much 18:2n-6. This variation could depend on both species and environmental differences. Storeheier *et al.* (31) showed that the nutritive value of lichens can differ greatly between species and within genera. Even though the lichens contained more n-3 LCPUFA than the pellets, the ratio of n-6/n-3 was relatively high due to a high proportion of 18:2n-6 in the lichens.

Earlier studies have shown that sex hormone status influenced FA composition in both adipose tissue and phospholipids (33,34) (reindeer and cattle). The present study however, showed in general no important effects of sex or age on the FA composition in the meat; indeed, we suggest the main differences depend on fatness, which is expressed by higher proportions of SFA in adult reindeer and lower proportions of PUFA in the calves.

In our reindeer study the proportions of both PL n-3 and n-6 PUFA do not differ between male and female animals, whereas the proportion of 22:6n-3 is significantly lower in female reindeer compared with males. However, the major differences in PL can be seen between males and calves in our study. In contrast to the findings in cows and yearlings of Malau-Aduli *et al.* (33), who analyzed phospholipids, we found higher proportions of PL n-3 in the meat from reindeer calves. The generally higher proportion of 22:5n-3 in PL fraction from calves could be a sign that calves accumulate this FA to a greater extent. Because 22:5n-3 is also increased in NL fraction from calves, this suggests a surplus intake and possibly also desaturation and elongation.

The higher proportion of NL PUFA in calves might be a

TABLE 4
FA Composition of Reindeer Calves Fed Different Types of Pellets Compared with Free-Ranging Calves (% of total identified FA)[a]

Polar lipids	CPD-fed (n = 7)	SD	LPD-fed (n = 10)	SD	Grazing (n = 7)	SD	P
16:0	10.9[ab]	0.67	11.1[b]	0.91	10.1[a]	0.72	0.035
17:0	0.21[a]	0.03	0.19[a]	0.03	0.29[b]	0.03	<0.001
18:0	14.3	0.66	14.9	0.60	14.3	0.74	0.122
12:1	0.06[a]	0.07	trace[b]		0.18[c]	0.03	<0.001
14:1	0.15	0.06	0.17	0.05	0.15	0.02	0.627
16:1*trans*	0.35[a]	0.05	0.46[b]	0.06	0.32[a]	0.04	<0.001
16:1n-9	1.11[a]	0.26	1.20[ab]	0.12	1.33[b]	0.15	0.077
17:1	0.16	0.07	0.15	0.07	0.15	0.04	0.994
18:1*trans*	0.50[a]	0.14	0.41[a]	0.14	0.11[b]	0.04	<0.001
18:1n-9	14.9[a]	2.20	16.2[a]	1.85	18.1[b]	1.14	0.010
18:1n-7	1.53	0.29	1.57	0.26	1.44	0.27	0.615
18:1n-5	0.21	0.04	0.20	0.04	0.21	0.03	0.764
18:2n-6	29.0[a]	2.85	27.2[a]	1.95	22.6[b]	1.01	<0.001
18:3n-6	0.14[a]	0.05	0.21[b]	0.03	0.13[a]	0.02	<0.001
18:3n-3	1.63[a]	0.43	2.31[b]	0.32	1.25[c]	0.22	<0.001
20:3n-6	1.06	0.12	1.02	0.10	1.05	0.09	0.728
20:3n-3	0.27[a]	0.04	0.25[ab]	0.04	0.32[b]	0.06	0.046
20:4n-6	14.2[a]	0.74	12.9[b]	1.19	17.0[c]	1.44	<0.001
20:5n-3	2.63[a]	0.59	2.89[ab]	0.27	3.22[b]	0.54	0.077
22:4n-6	0.74	0.13	0.70	0.08	0.80	0.18	0.252
22:5n-3	5.27[a]	0.45	5.31[a]	0.29	6.03[b]	0.42	0.001
22:6n-3	0.38[a]	0.05	0.37[a]	0.05	0.51[b]	0.03	<0.001
Neutral lipids							
14:0	1.67	0.19	1.63	0.29	1.59	0.18	0.823
16:0	26.7	1.73	26.0	2.14	26.2	2.02	0.770
17:0	0.88	0.06	0.92	0.12	0.97	0.10	0.278
18:0	22.2	1.55	22.8	1.51	21.7	1.58	0.370
20:0	0.22[a]	0.03	0.20[a]	0.03	0.35[b]	0.13	0.001
12:1	0.81[a]	0.28	0.81[a]	0.26	1.13[b]	0.14	0.027
16:1*trans*	0.51[a]	0.05	0.51[a]	0.08	0.84[b]	0.21	<0.001
16:1n-9	1.50	0.17	1.50	0.20	1.56	0.17	0.760
17:1	0.21[a]	0.04	0.19[a]	0.04	0.25[b]	0.04	0.015
18:1*trans*	1.43[a]	0.34	1.18[a]	0.34	0.48[b]	0.06	<0.001
18:1n-9	39.3	1.58	40.0	2.08	39.2	1.91	0.659
18:1n-7	1.04[a]	0.13	0.92[a]	0.07	1.22[b]	0.25	0.003
20:1n-9	0.26[ab]	0.03	0.23[a]	0.03	0.28[b]	0.07	0.108
18:2n-6	2.09	0.37	1.88	0.21	1.99	0.46	0.491
18:3n-3	0.26[ab]	0.12	0.32[a]	0.09	0.18[b]	0.04	0.017
20:4n-6	0.20[a]	0.03	0.19[a]	0.05	0.46[b]	0.20	<0.001
22:5n-3	0.20[a]	0.05	0.29[a]	0.10	1.05[b]	0.78	0.002
Total lipids(%)	2.49	0.31	2.52	0.56	2.10	0.46	0.129

[a]Means with different superscripts within a row differ significantly ($P < 0.05$). FA are presented if at least one group had values > 0.15%. In total 32 FA were identified.

result of less dilution in adult animals by lower *de novo* synthesis of PUFA. Also, due to the higher demand for energy due to growth, lower proportions of SFA are deposited in calves. Similar results have also been reported in lambs; older lambs had higher SFA and lower n-6 content in total lipids compared with younger ones (35). However, differences in FA composition were correlated with higher fatness in females compared with males and with increasing age (36) (pigs, cattle, and lamb).

The fact that 12:0 was found in relatively high proportions in the pellets (7.05% in CPD and 6.08% in LPD), but not in the meat lipids, indicates that the reindeer use this FA as an energy source or store it in the adipose tissue and do not store it in the muscles. The differences in n-6, SFA, and MUFA in the PL fraction between the pellet-fed and the grazing animals are probably due to the different proportion of these FA in the pellets compared to the lichens and the other feed items consumed. This is especially true for the LCPUFA 20:4n-6, 20:5n-3, 22:5n-3, and 22:6n-3, which are not present in the pellets. The natural feed of grazing animals significantly affects the composition of the PL, which is exhibited by the FA mentioned above. These FA are significantly higher in the PL fraction of meat from the grazing animals compared with the pellet-fed animals. It is suggested that this difference is caused by the difference in feed composition. Similar results have been found in an earlier study on grazing and pellet-fed reindeer (5).

However, some lichen FA were not reflected in the meat in the same proportions as the pellet FA were. For example, 18:2n-6 was lower in meat from grazing reindeer than pellet-

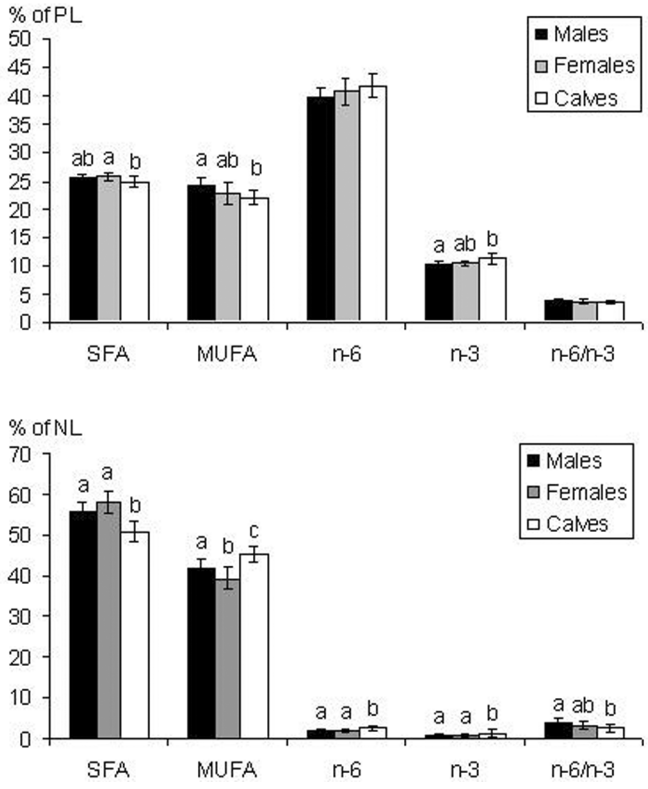

FIG. 1. FA composition of free-ranging reindeer of different age and sex (percent of total identified FA; mean values and standard deviation) presented as sums of saturated and unsaturated FA. Means with different superscripts within a row differ significantly ($P < 0.05$).

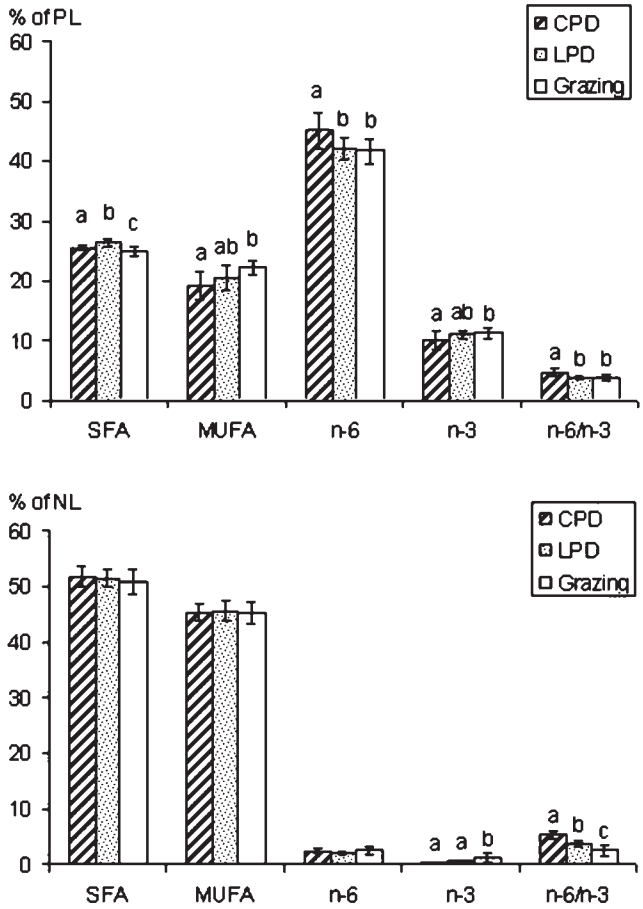

FIG. 2. FA composition of reindeer calves fed different types of pellets compared with free-ranging calves (percent of total identified FA; mean values and standard deviation) presented as sums of saturated and unsaturated FA. Means with different superscripts within a row differ significantly ($P < 0.05$).

fed ones in spite of the higher proportion in the analyzed lichens compared with the pellets. Grazing of green plants other than lichens possibly caused a lower intake of 18:2n-6. Lichens are estimated to contribute to 13–56% (31,37–39) or even 50–80% (40) of the winter diet. Green feed, such as grass, contains 14.7% 16:0 and 10% 18:2n-6, but up to 68% 18:3n-3 (41). This composition illustrates well the general green plant FA profile.

As the PL have a relatively stable composition due to their functionality, they are in general less affected, but variation can be found within the groups of SFA and unsaturated FA, for example, if one PUFA is increased another similar one will be decreased to balance this (15,42) (fish, cattle). Nonetheless the significantly higher proportion of 18:3n-3 in the LPD is reflected in the PL fraction in addition to a significantly higher proportion of 18:2n-6. In the present study the higher proportions of 18:2n-6 and 18:3n-3 in LPD-fed reindeer are probably balanced by lower contents of 20:4n-6 and 22:5n-3 as argued by Scollan *et al.* (15) (cattle).

The increased amount of 20:5n-3 in the meat of the LPD-fed animals compared with the CPD-fed reindeer group indicates an enhanced synthesis from 18:3n-3; similar results were also found in a study of beef (15). However, this does not apply for 22:5n-3 and 22:6n-3 in the LPD-fed animals. The n-3 LCPUFA were still somewhat lower in the LPD-fed reindeer compared with the grazing reindeer, even though the total n-3 in PL of the meat from the animals fed LPD did not differ significantly from the grazing animals. This suggests either that the increase of 18:3n-3 was still too small to enhance the synthesis of longer-chain FA or that the animals are not able to elongate and desaturate these FA in significant amounts. A similar limited elongation and desaturation is found in pigs (43) and rats (44). This lack of elongation and the fact that 22:6n-3 competes poorly against 20:5n-3 for incorporation into PL indicates that the reindeer need to ingest LCPUFA *via* the diet. It can be argued that even a low amount of essential FA and their elongated and desaturated derivatives are accumulated in the food chain and therefore of significant importance.

The grazing reindeer had a slightly lower content of retinol than the pellet-fed animals, but the difference was not as large as the difference in the feed, which suggests that the animals are not able to take up and metabolize higher amounts of retinol.

γ-Tocopherol was significantly higher in the pellet-fed reindeer, which correlates with the significantly higher

TABLE 5
Vitamin Content (μg/g) in Meat from Free-Ranging Reindeer of Different Age and Sex[a]

	Males (n = 7)	SE	Females (n = 6)	SE	Calves (n = 7)	SE	P
Retinol	0.026	0.003	0.030	0.003	0.029	0.003	0.648
γ-Tocopherol	0.035	0.005	0.031	0.005	0.025	0.005	0.441
α-Tocopherol	4.47[a]	0.288	4.90[a,b]	0.311	5.67[b]	0.288	0.028

[a]Values reported are least-square means and standard errors. Means with different superscripts within a row differ significantly ($P < 0.05$).

TABLE 6
Vitamin Content (μg/g) in Meat from Reindeer Calves Fed Different Types of Pellets Compared with Free-Ranging Calves[a]

	CPD-fed (n = 7)	SE	LPD-fed (n = 10)	SE	Grazing (n = 7)	SE	P
Retinol	0.039[a]	0.003	0.045[a]	0.002	0.029[b]	0.003	0.001
γ-Tocopherol	0.055[a]	0.009	0.067[a]	0.007	0.025[b]	0.009	0.002
α-Tocopherol	5.50	0.293	5.36	0.245	5.67	0.293	0.722

[a]Values reported are least-square means and standard errors. Means with different superscripts within a row differ significantly ($P < 0.05$). CPD, conventional pellet diet; LPD, linseed pellet diet.

amount of γ-tocopherol in the pellets compared to the analyzed lichens. In the lichens γ-tocopherol was only detected in small amounts in *Cladina mitis*; it was not detectable in the other lichen species included in this study.

Otherwise, the difference in tocopherols between the pellets, which contain 56.5 and 48.4 μg/g tocopherol acetate in CDP and LDP, respectively, and the lichens, which contain much lower amounts of total tocopherols (9.4–15.8 μg/g α-tocopherol), is not reflected in the meat. This can be explained by several mechanisms. First, it might be that the animals reach a saturation level as shown for cattle (45). Also Jensen *et al.* (46) present data from several trials on pigs and poultry in which vitamin E increases only slightly with increased supplementation. Second, it is possible that some substances in the lichens increase tocopherol bioavailability, as it was suggested that phenolic compounds affect tocopherol levels in tissues (47) (rats and humans).

In addition, the natural antioxidants can have an additive value for the total antioxidant capacity. These mechanisms are still very much unknown. Gülçin *et al.* (48) showed that aqueous lichen extract inhibited lipid peroxidation to a higher extent than α-tocopherol in a linoleic acid emulsion, which indicates that one or more other compounds with antioxidant function might be found in lichens. Therefore we suggest that α-tocopherol might accumulate in the meat because it is not affected by nonenzymatic oxidation.

The consumption of reindeer meat is traditionally relatively high among Sami reindeer herders compared with people of Swedish, Finnish, and Norwegian origin living in the same areas (49,50). Reindeer meat FA composition is suggested to influence the n-6/n-3 ratio and the intake of LCPUFA and thereby affect health. Studies of health among reindeer herders showed a significantly lower incidence of ischemic heart disease in Finnish Sami compared with Finns (51), which could be related to diet, but may also depend on other lifestyle factors. These results were suggested to be related to the diet, especially the consumption of reindeer meat. The high content of tocopherol increased serum α-tocopherol, and the FA composition was suggested to beneficially affect thrombogenesis and reduce atherogenicity (51).

CONCLUSION

Effects on FA composition of reindeer meat were found to be mainly diet dependent. Tocopherol and retinol contents were only slightly affected by different feeding regimen or sex.

Feeding reindeer pelleted feed containing crushed linseed positively affects the FA composition and decreases the n-6/n-3 ratio. It is possible through the feed to influence the FA composition of the meat toward a more nutritionally valuable composition. The animals are not able to fully elongate and desaturate linolenic acid toward 22:5n-3 and 22:6n-3, which indicates that reindeer need to ingest these FA via the diet.

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Year-round High Arachidonic Acid Levels in Herbivorous Rabbit Fish *Siganus fuscescens* Tissues

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ABSTRACT: To identify a stable resource of 20:4 n-6 (arachidonic acid, AA) in marine fish tissues, the lipid profiles of *Siganus fuscescens* organs (muscle, liver, and other viscera) and stomach contents were examined throughout the year. Crude total lipid (TL) contents in respective organs showed seasonal variations and were high in winter and low in summer. The main FA in TL were 16:0, 18:0, 16:1n-7, 18:1n-9, AA, and 22:6n-3 (DHA). These FA were those generally observed in marine fish lipids, except for comparatively high levels of AA. In TL of muscle and liver, AA showed relatively high values during the period from late May to August (muscle, 4.6–13.1%; liver, 4.5–9.1%), compared with other seasons (muscle, 4.3–9.5%; liver, 3.6–8.4%). The AA levels in TL of other viscera and stomach contents fluctuated (other viscera, 2.0–10.7%; stomach contents, 7.6–26.7%). Regardless of the fishing season, each organ contained a higher level of AA in polar lipids (PL) than in neutral lipids. It was concluded that the fish contain comparatively high levels of AA in their TL throughout the year, and they accumulate AA characteristically in their tissue PL, probably from dietary food sources. Moreover, it was suggested that *S. fuscescens* has potential utility as a natural marine source of nutritional lipids, because the fish contain comparatively high levels of DHA and AA.

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The desirable biochemistry of fish lipids as part of the human diet has been confirmed. In particular, many reports indicate that n-3 series PUFA are useful for suppressing increases in plasma cholesterol (1,2), preventing cardiovascular diseases (3–5), and improving learning ability and visual function (6,7). Consequently, for the purpose of developing sources of n-3 series PUFA, especially DHA, scientists have been studying the chemistry of fish lipids.

Generally, human beings are able to synthesize arachidonic acid (AA) or DHA by chain elongation and desaturation from linoleic acid (18:2n-6) or alpha-linolenic acid, but this process is inefficient, particularly in infants (8,9).

The importance of the intake balance of DHA and AA for human health, especially for the growth of infants, has only recently been clarified (10–12). Therefore, intake of AA with DHA is presumed to be essential for the growth of infants.

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Abbreviations: AA, arachidonic acid; DMOX, 4, 4-dimethyloxazoline; GSI, gonadsomatic index; MUFA, monounsaturated FA; NL, neutral lipids; PL, polar lipids; SFA, saturated FA; ST, sterols; TFA, total FA; TL, total lipids; WE, wax esters.

The marine resource of DHA has been gradually identified, and an industrial supply system has been established. Nevertheless, no information is available on AA from the viewpoint of natural resources.

Industrially, AA is produced by extracting oil from the bacterium *Mortierella alpina* (13), and is used as a dietary supplement or food additive. However, in Japan, AA produced by this method has not been allowed for use as an additive in infant formula (modified milk) by the Ministry of Health, Labour and Welfare. Therefore, a natural, stable resource of AA is needed.

Generally, freshwater fish have high levels of n-6 PUFA in their lipids, from terrestrial prey rich in n-6 PUFA that they commonly consume (14–16). In contrast, marine fish lipids contain n-3 PUFA originating from phytoplankton through the marine food chain, whereas the levels of n-6 PUFA, such as AA, in their lipids are undetectable or negligible. However, there are several marine species whose lipids contain comparatively high levels of n-6 PUFA, including some kinds of algae, abalone, and fish (17–22).

Herbivorous species (including omnivorous species) reportedly contain high levels of AA (23–25). Moreover, carnivorous fishes that feed on macro-algae-consuming sea urchins and sea snails are also known to have high levels of AA (25). The high levels of AA observed in these species may originate in macroalgae, which generally contain high levels of AA. However, there are no reports that discussed the relationships between AA levels in fish organs and their diets, except for the report by Saito *et al.* (22).

Siganus fuscescens (Houttuyn, 1782) is one of the most common herbivorous fish species in Japan. This fish is abundantly caught as a by-catch species year-round, and their excessive consumption of algae is known as the main cause of the subtidal barren observed at the Japanese offshore line (26–29). This fish could be useful as a natural, stable source of both AA and DHA if it contains both of these PUFA abundantly in its tissues.

It has been reported that the total FA (TFA) composition of several fish species varies from season to season due to environmental conditions, including its natural cycle, maturity stage, geographic location, and other factors (30–37). However, there are no reports on seasonal variations in the FA composition of herbivorous fish such as *S. fuscescens*.

We assessed the FA composition of a typical herbivorous fish, *S. fuscescens*, throughout the year to analyze seasonal variations.

EXPERIMENTAL PROCEDURES

Fish. S. fuscescens were caught using set nets between April 11, 2002, and September 29, 2004, in the Nagasaki offshore (33°19'N, 129°02'E), Japan, and transported to our laboratory under ice-storage condition. The fork length and body weight of the fish were 24.9–34.7 cm and 273.4–502.2 g, respectively.

Preparation of sample for lipid analyses. The fish were measured for fork length and body weight. Dorsal ordinary muscles, liver, and other viscera (without stomach contents, includes stale tissues) were then cut out using a scalpel after wiping. The stomach contents were then removed and used as samples.

Lipid extraction and analyses of lipid classes. Individual tissue samples were minced and homogenized with 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. (38). The total lipids (TL) of specimens collected on November 27, 2002; July 30, 2003; and August 1, 2003 were separated into classes on silicic acid columns, and the constituent lipids were quantified by gravimetric analyses of column chromatographic fractions. The first eluate (dichloromethane/ether, 9:1, vol/vol) was used to collect the neutral lipids (NL). The second elute (dichloromethane/methanol, 10:1, vol/vol) from the column contained the free FA (FFA). This was followed with dichloromethane/methanol (1:20, vol/vol), eluting polar lipids (PL). Individual lipids, separated from each lipid class, were identified by comparing with standard samples using TLC (thickness of 0.25 mm for analysis, Kiesel gel 60, Merck and Co., Ltd., Darmstadt, Germany). All sample lipids were dried under nitrogen at room temperature and stored at –70°C with a small amount of dichloromethane.

The preparation of methyl esters and GLC of the esters. The TL, NL, and PL fractions were directly transesterified with boiling methanol containing 1% of concentrated hydrochloric acid under reflux for 1.5 h to produce the FAME (39). These methyl esters were purified using silica gel column chromatography by elution with dichloromethane.

Analysis of the FAME was performed on a gas chromatograph (GC–17A, Shimadzu Seisakusho Co., Ltd., Kyoto, Japan) equipped with a capillary column (Omegawax–250, 30 m × 0.25 mm i.d., 0.25 μm film thickness; split ratio 10:1; Supelco Japan Co., Ltd., Tokyo, Japan). The temperatures of the injector, column, and detector were maintained at 290, 205, and 290°C, respectively. Helium was used as the carrier gas at a constant inlet rate of 17 mL/min.

Quantitation of individual components was performed by means of Shimadzu Model C-R7A (Shimadzu Seisakusho Co. Ltd.) electronic integrator. The weight percentages of individual components in the lipids were expressed as relative percentage of peak area to total peak area.

The preparation of 4,4-dimethyloxazoline (DMOX) derivatives and analysis by GC–MS. The DMOX derivatives were prepared by adding excess 2-amino-2-methylpropanol to a small amount of the FAME in a test tube under nitrogen atmosphere. The mixture was heated at 180ºC for 18 h. The reaction mixture was cooled and poured onto saturated brine and extracted with n-hexane. Triplicate extraction with n-hexane was carried out, and the extract was washed with saturated brine, and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and samples were dissolved again in n-hexane for analysis by GC–MS (40).

Analysis of the DMOX derivatives was performed on a GC-MS QP 2010 (Shimadzu Seisakusho Co., Ltd.) gas chromatograph–mass spectrometer equipped with the same capillary column described in the previous section. The temperatures of the injector and the column were held at 240 and 210ºC, respectively. The split ratio was 1:76, and the ionization voltage was 70 eV. Helium was used as the carrier gas at a constant inlet rate of 0.7 mL/min.

FAME were identified (i) using marine lipid methyl esters as standards (Supelco 37 Component FAME Mix, Supelco Japan Ltd.), and (ii) by comparison of mass spectral data obtained by GC–MS.

Statistical analyses. Averages were compared based on one-way ANOVA (Statview, Version 5, SAS Institute Inc., Cary, NC). Values were considered statistically different at $P < 0.05$.

RESULTS AND DISCUSSION

Seasonal variations in gonadsomatic index (GSI) and TL in respective tissues. The body size, GSI, and lipid content of respective organs of sampled fish are presented in Table 1. The high GSI values from June to August (2.7–10.2) compared with other seasons (0.6–1.7) indicated that the spawning period of the fish occurs during June to August. A previous report on the spawning season of the species for the same area confirmed this finding (41).

Muscle TL levels showed seasonal variation, with relatively high values (1.7 ± 0.5%) from September to January, and low values (1.1 ± 0.4%) from February to August, although the variations were not as clear as those in visceral TL. The levels of TL in both liver and other viscera varied seasonally in a clear manner, with high values in October and November (liver, 7.0–18.0%; other viscera, 25.1–37.3%), decreased values from November to late May (liver, 4.9–18.0%; other viscera, 4.1–37.3%), and low values from June to August (liver, 2.2–4.8%; other viscera, 1.3–6.0%). Generally, muscle TL of pelagic fishes varies widely, because they store their depot lipids in this tissue (42,43), whereas demersal fishes use viscera as a lipid storage site (44,45). *S. fuscescens* shows the typical character of demersal species.

Sexual maturation has been found to reduce lipids stored in the body of salmon because this species stops feeding during maturation and uses stored lipid as an energy source for spawning (46). Other studies showed that reduced feeding level and sexual maturation contribute to reduced lipid levels (47). Many marine fish species tend to reduce their food intake during sexual maturation; lipids and other nutrients needed for gonadal growth are presumed to be taken from the reserves in their bodies.

TABLE 1
Body Size, GSI, and Lipid Content of Organs of Sampled *S. fuscescens*[a,b]

Date	Sample size	Fork length (cm)	Body weight (g)	GSI[c]	Lipid contents (%)		
					Muscle	Liver	Other viscera
2002							
April 11	(*n* = 5)	30.4 ± 1.1	442.6 ± 52.1	1.0 ± 0.3	2.1 ± 0.8	7.1 ± 2.8	24.3 ± 12.0
April 23	(*n* = 5)	29.0 ± 0.7	365.3 ± 17.6	1.0 ± 0.2	0.9 ± 0.5	4.6 ± 1.1	11.5 ± 7.3
May 25	(*n* = 2)	29.4 ± 2.5	467.9 ± 105.7	1.3 ± 0.2	1.0 ± 0.2	4.9 ± 0.1	4.1 ± 0.5
June 6	(*n* = 2)	29.5 ± 1.7	449.7 ± 82.9	6.4 ± 4.5	0.9 ± 0.5	3.8 ± 0.3	4.7 ± 0.9
July 9	(*n* = 5)	25.8 ± 0.4	329.3 ± 13.4	8.8 ± 2.2	1.1 ± 0.6	4.4 ± 1.7	6.0 ± 2.2
August 1	(*n* = 3)	25.9 ± 0.1	273.4 ± 16.9	7.2 ± 2.0	1.3 ± 0.5	4.8 ± 0.8	2.6 ± 0.9
August 7	(*n* = 5)	26.3 ± 0.8	289.1 ± 29.8	5.3 ± 1.0	0.8 ± 0.3	4.4 ± 1.2	2.5 ± 1.2
September 30	(*n* = 5)	27.8 ± 1.4	306.9 ± 27.1	0.3 ± 0.1	0.9 ± 0.5	4.3 ± 1.0	17.2 ± 5.5
October 16	(*n* = 5)	25.4 ± 0.3	293.2 ± 20.3	0.2 ± 0.1	1.8 ± 0.7	7.0 ± 1.0	25.1 ± 3.2
November 8	(*n* = 4)	26.2 ± 0.6	340.4 ± 16.0	0.1 ± 0.0	2.7 ± 1.1	10.3 ± 3.5	37.1 ± 7.4
November 27	(*n* = 5)	26.0 ± 0.6	313.2 ± 14.4	0.4 ± 0.1	1.5 ± 1.0	18.0 ± 13.9	37.3 ± 16.9
December 19	(*n* = 5)	29.2 ± 0.8	382.5 ± 15.4	0.5 ± 0.2	1.1 ± 0.7	4.9 ± 0.8	29.5 ± 14.4
2003							
April 10	(*n* = 5)	26.2 ± 0.4	304.3 ± 19.0	0.4 ± 0.1	1.4 ± 0.4	5.1 ± 1.8	21.5 ± 11.2
August 1	(*n* = 3)	34.7 ± 1.7	502.2 ± 20.5	10.2 ± 4.9	0.5 ± 0.1	2.2 ± 0.6	2.3 ± 0.5
November 18	(*n* = 5)	25.7 ± 0.7	310.9 ± 23.3	0.4 ± 0.1	1.7 ± 0.3	7.3 ± 1.8	31.3 ± 3.7
2004							
January 9	(*n* = 5)	30.0 ± 0.6	381.7 ± 9.7	0.6 ± 0.2	1.3 ± 0.7	6.0 ± 0.7	19.9 ± 9.7
March 11	(*n* = 6)	29.9 ± 0.7	376.1 ± 16.9	0.9 ± 0.1	0.9 ± 0.5	6.3 ± 1.4	12.9 ± 8.1
April 21	(*n* = 8)	24.9 ± 2.0	298.6 ± 90.4	0.6 ± 0.1	1.1 ± 0.5	6.4 ± 1.4	11.8 ± 9.1
May 22	(*n* = 3)	30.8 ± 1.9	470.3 ± 63.7	1.7 ± 0.1	1.5 ± 0.4	5.7 ± 0.7	13.6 ± 6.2
June 18	(*n* = 3)	30.2 ± 2.9	414.7 ± 122.0	2.7 ± 1.3	0.9 ± 0.5	4.4 ± 1.9	4.5 ± 2.5
July 30	(*n* = 3)	26.4 ± 1.6	288.1 ± 43.4	10.2 ± 4.0	0.6 ± 0.1	4.0 ± 1.4	1.3 ± 0.7
February 17	(*n* = 5)	30.1 ± 1.0	372.0 ± 24.5	0.6 ± 0.2	0.9 ± 0.2	7.6 ± 3.4	17.7 ± 2.7
September 29	(*n* = 6)	29.5 ± 2.2	450.9 ± 88.5	0.3 ± 0.1	2.1 ± 0.7	10.1 ± 4.0	35.3 ± 8.0

[a]Lipid contents are expressed as weight percentage of wet tissues.
[b]Data are mean ± SE.
[c]GSI is the mean percentage of gonad weight to body weight.

In individuals caught off the Japanese coast in the Nagasaki district, the minimum levels of lipid observed in summer may indicate reduced feeding and lipid consumption as an energy source for spawning. The increase of lipid level in winter may be explained by enhanced feeding activity for recovery and growth.

FA composition of TL in tissues and stomach contents. FA compositions of TL in specific organs are presented in Tables 2–4. The FA compositions were almost the same among the organs, and the major components (>5% in annual average) of muscle were 16:0 (muscle, 21.2–30.7%; liver, 25.0–36.6%; other viscera, 22.9–34.0%), 18:0 (muscle, 5.2–9.3%; liver, 4.4–9.0%; other viscera, 5.0–8.2%), 16:1n-7 (muscle, 2.7–15.4%; liver, 4.3–13.9%; other viscera, 4.5–16.6%), 18:1n-9 (muscle, 7.0–15.8%; liver, 5.6–18.5%; other viscera, 8.0–20.2%), 20:4n-6 (AA; muscle, 4.3–13.1%; liver, 3.6–9.1%; other viscera, 2.0–10.7%), and 22:6n-3 (DHA; muscle, 8.0–22.4%; liver, 7.7–22.1%; other viscera, 2.9–17.1%). Moderate amounts (>2% in annual average) of other FA, such as n-6 PUFA 22:5 n-6 (muscle, 1.4–4.1%; liver, 1.1–3.7%; other viscera, 0.6–2.9%), n-3 PUFA 20:5n-3 (muscle, 1.2–4.2%; liver, 0.4–3.1%; other viscera, 0.5–3.7%), and 22:5n-3 (muscle, 2.5–6.2%; liver, 1.3–5.0%; other viscera, 1.6–6.6%), were also detected.

The comparatively high AA levels in *S. fuscescens* organs were similar to those reported in other herbivorous species including *Caesio diagramma*, *C. tile*, and *S. canaliculatus*, as reported by Saito *et al.* (22). Thus, the comparatively high levels of AA may be a specific characteristic of herbivorous species. The FA compositions in stomach contents are shown in Table 5. The stomach contents were a mixture of small pieces of seaweed, mucoid substance, sludge, and other components. The major FA in stomach contents TL were 16:0 (12.5–27.2%), 18:0 (6.6–14.8%), 18:1n-9 (3.5–9.8%), AA (7.6–26.7%), and DHA (8.8–20.3%), similar to those in the tissue TL of *S. fuscescens*. These findings suggest that the high levels of AA in *S. fuscescens* originated from their food sources.

Seasonal variations in the FA composition of TL in tissues and stomach contents. In the muscle TL, the total SFA levels showed constant values of 37.1 ± 0.5% (annual average ± SE) throughout the year, and those of MUFA differed somewhat between late May to August (13.6–29.0%) and other seasons (17.9–34.9%). Total PUFA levels showed seasonal tendencies opposite to those of total MUFA levels. In the liver TL, total SFA levels were almost constant throughout the year at 40.4 ± 0.8%. Similar seasonal variations to those in muscle TL were found in liver TL, where total MUFA levels were low from late May to August (13.3–21.9%) and higher during the rest of the

TABLE 2
FA Composition of *S. fuscescens* Muscle[a,b]

	2002												2003
	Apr. 11	Apr. 23	May 25	Jun. 6	Jul. 9	Aug. 1	Aug. 7	Sept. 30	Oct. 16	Nov. 8	Nov. 27	Dec. 19	Apr. 10
Total saturated	39.7 ± 1.3	37.6 ± 1.4	36.2 ± 0.7	35.3 ± 1.1	36.6 ± 0.4	34.3 ± 1.4	39.1 ± 3.7	41.5 ± 0.9	41.1 ± 0.7	37.4 ± 2.8	36.6 ± 1.5	37.2 ± 1.9	36.2 ± 1.1
12:0	0.6 ± 0.1	0.5 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
14:0	3.2 ± 0.2	3.6 ± 0.4	2.9 ± 0.0	2.7 ± 0.7	2.7 ± 0.3	2.9 ± 0.4	2.3 ± 0.3	3.3 ± 0.5	3.2 ± 0.1	2.5 ± 0.4	2.7 ± 0.1	3.1 ± 0.6	2.6 ± 0.4
15:0	0.3 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1
16:0	28.7 ± 0.9	26.3 ± 0.9	24.8 ± 0.7	24.8 ± 1.1	24.7 ± 0.5	22.2 ± 1.4	25.1 ± 2.3	30.7 ± 1.0	29.8 ± 0.6	26.7 ± 2.9	25.9 ± 1.0	26.4 ± 1.7	25.7 ± 0.8
17:0	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.9 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	1.1 ± 0.2	0.6 ± 0.1	0.4 ± 0.0
18:0	5.3 ± 0.3	5.2 ± 0.4	6.7 ± 0.1	6.4 ± 0.9	6.6 ± 0.6	7.1 ± 0.4	9.3 ± 1.5	6.4 ± 0.7	6.5 ± 0.1	6.7 ± 0.3	5.7 ± 0.5	5.7 ± 0.6	6.3 ± 0.3
19:0	0.9 ± 0.2	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
20:0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.2 ± 0.0	0.2 ± 0.0
Total monoenoic	34.9 ± 3.0	29.0 ± 3.3	17.8 ± 0.6	23.0 ± 4.5	18.0 ± 1.6	21.9 ± 2.6	17.0 ± 2.2	22.3 ± 1.6	30.1 ± 2.0	28.1 ± 3.4	32.3 ± 2.6	26.8 ± 3.6	22.1 ± 2.0
16:1n-7	15.4 ± 1.1	11.2 ± 1.9	5.5 ± 0.1	6.5 ± 2.2	6.5 ± 1.3	7.1 ± 1.4	5.0 ± 1.1	9.1 ± 1.3	10.1 ± 0.6	8.7 ± 1.1	12.0 ± 0.7	9.9 ± 2.1	7.4 ± 1.0
18:1n-9	13.9 ± 1.8	13.0 ± 1.3	8.9 ± 0.6	12.6 ± 1.6	7.0 ± 0.4	10.2 ± 1.4	8.5 ± 0.9	9.7 ± 0.9	15.3 ± 1.2	15.1 ± 2.1	12.5 ± 1.2	12.6 ± 1.2	10.9 ± 0.9
18:1n-7	4.7 ± 0.8	3.6 ± 0.4	2.4 ± 0.1	2.8 ± 0.4	3.4 ± 0.4	3.4 ± 0.0	2.8 ± 0.2	2.9 ± 0.1	3.9 ± 0.3	3.2 ± 0.3	6.6 ± 0.7	3.2 ± 0.4	2.8 ± 0.2
18:1n-5	0.3 ± 0.1	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.2	0.1 ± 0.0	0.2 ± 0.1
20:1n-11	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1
20:1n-9	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	0.2 ± 0.0	0.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0
≤22:1n-11	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.5 ± 0.3	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:1n-9	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
24:1n-9													
Total polyenoic													
n-6 series	8.7 ± 0.6	9.4 ± 1.3	12.4 ± 0.2	12.5 ± 2.3	11.8 ± 0.9	10.7 ± 0.9	12.6 ± 1.3	11.2 ± 1.3	8.8 ± 0.7	10.4 ± 1.4	9.2 ± 1.3	12.2 ± 2.7	13.1 ± 1.5
18:2n-6	1.5 ± 0.4	1.0 ± 0.2	0.9 ± 0.1	1.2 ± 0.0	1.6 ± 0.5	1.1 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.5 ± 0.3	1.2 ± 0.2	1.3 ± 0.3	1.1 ± 0.2	1.2 ± 0.3
18:3n-6	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.4 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
20:2n-6	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
20:3n-6	0.3 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.1
20:4n-6	4.5 ± 0.5	4.9 ± 0.8	7.2 ± 0.1	6.4 ± 1.4	6.9 ± 1.0	5.7 ± 0.5	7.3 ± 0.9	6.6 ± 0.8	4.3 ± 0.6	5.8 ± 1.0	5.1 ± 0.9	6.7 ± 1.8	7.4 ± 1.0
22:4n-6	0.7 ± 0.1	1.0 ± 0.1	1.4 ± 0.0	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.2 ± 0.2	1.1 ± 0.1
22:5n-6	1.5 ± 0.2	1.7 ± 0.2	2.1 ± 0.0	2.7 ± 0.5	1.6 ± 0.3	2.2 ± 0.3	2.8 ± 0.2	1.9 ± 0.3	1.4 ± 0.1	1.7 ± 0.4	1.5 ± 0.3	2.4 ± 0.7	2.7 ± 0.6
n-3 series	12.7 ± 3.2	18.1 ± 2.0	28.3 ± 1.1	21.9 ± 2.6	27.4 ± 1.2	27.1 ± 3.2	26.1 ± 2.2	18.1 ± 1.5	15.1 ± 2.0	16.8 ± 2.9	16.4 ± 1.4	18.3 ± 2.7	24.4 ± 2.0
18:3n-3	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	1.2 ± 0.4	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
18:4n-3	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.9 ± 0.3	0.2 ± 0.0	0.3 ± 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
20:3n-3	0.0 ± 0.0	0.0 ± 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	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.0	0.3 ± 0.1	0.9 ± 0.2	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
20:5n-3	1.4 ± 0.5	1.9 ± 0.4	3.1 ± 0.2	2.2 ± 0.5	4.2 ± 0.3	1.7 ± 0.0	2.1 ± 0.2	2.0 ± 0.2	1.3 ± 0.3	1.9 ± 0.4	2.0 ± 0.4	1.7 ± 0.3	2.6 ± 0.2
22:5n-3	2.5 ± 0.7	3.8 ± 0.4	6.2 ± 0.7	2.8 ± 0.7	5.7 ± 0.2	4.7 ± 0.2	4.4 ± 0.4	3.3 ± 0.1	2.7 ± 0.4	2.9 ± 0.3	2.7 ± 0.3	4.0 ± 0.6	4.1 ± 0.1
22:6n-3	8.0 ± 2.0	11.2 ± 1.7	17.6 ± 0.2	16.2 ± 2.9	14.3 ± 1.9	19.7 ± 3.0	18.5 ± 1.8	11.6 ± 1.6	9.8 ± 1.3	10.9 ± 2.2	10.7 ± 1.2	11.4 ± 2.3	16.5 ± 2.1

[a]Results are expressed as weight percentage of total FA.
[b]Data are mean ± SE.

TABLE 2 (continued)
FA Composition of *S. fuscescens* Muscle[a,b]

	2003		2004							
	Aug. 1	Nov. 18	Jan. 9	Feb. 17	Mar. 11	Apr. 21	May 22	Jun. 18	Jul. 30	Sept. 29
Total saturated	32.3 ± 1.2	39.6 ± 1.1	38.7 ± 2.2	39.1 ± 0.8	36.6 ± 1.2	35.0 ± 0.4	38.5 ± 0.5	33.7 ± 2.9	36.3 ± 0.9	35.2 ± 2.4
12:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
14:0	1.0 ± 0.1	3.1 ± 0.1	3.3 ± 0.6	3.0 ± 0.2	2.0 ± 0.3	2.2 ± 0.1	3.1 ± 0.1	2.8 ± 0.8	2.4 ± 0.6	3.6 ± 0.6
15:0	0.7 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.0
16:0	21.2 ± 1.0	28.6 ± 1.6	27.5 ± 2.2	27.6 ± 1.0	26.1 ± 1.2	24.5 ± 0.4	27.6 ± 0.6	23.8 ± 2.6	25.2 ± 0.9	24.5 ± 1.8
17:0	0.5 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
18:0	8.4 ± 0.1	6.3 ± 0.2	6.6 ± 0.6	6.8 ± 0.3	7.4 ± 0.6	6.8 ± 0.2	6.5 ± 0.4	5.4 ± 0.5	7.2 ± 0.6	5.4 ± 0.5
19:0	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0
20:0	0.1 ± 0.0	0.3 ± 0.1	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
Total monoenoic	13.6 ± 0.9	29.4 ± 2.4	24.4 ± 3.1	25.1 ± 2.4	21.2 ± 2.4	17.9 ± 0.7	29.0 ± 1.3	21.1 ± 3.2	17.9 ± 4.0	27.9 ± 2.8
16:1n-7	2.7 ± 0.4	9.5 ± 1.2	8.3 ± 1.4	8.0 ± 0.7	6.2 ± 1.3	5.2 ± 0.2	8.8 ± 0.8	8.8 ± 2.5	6.3 ± 2.7	7.9 ± 1.1
18:1n-9	7.6 ± 0.5	15.0 ± 1.4	11.7 ± 1.6	13.4 ± 1.5	11.7 ± 0.9	9.3 ± 0.7	15.8 ± 0.4	8.6 ± 0.3	7.4 ± 0.9	14.0 ± 2.1
18:1n-7	2.3 ± 0.1	3.9 ± 0.1	3.3 ± 0.4	2.9 ± 0.1	2.6 ± 0.3	2.7 ± 0.1	3.5 ± 0.1	2.8 ± 0.3	3.9 ± 0.3	3.4 ± 0.2
18:1n-5	0.1 ± 0.0	0.1 ± 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	0.0 ± 0.0	0.1 ± 0.0
20:1n-11	0.1 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	1.0 ± 0.3
20:1n-9	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.6 ± 0.2
22:1n-11	0.3 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.5 ± 0.2
22:1n-9	0.3 ± 0.3	0.1± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
24:1n-9	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
Total polyenoic	50.0 ± 2.1	24.9 ± 2.5	31.5 ± 5.2	30.7 ± 3.0	38.0 ± 3.8	42.5 ± 0.8	26.2 ± 1.3	39.2 ± 5.7	41.2 ± 5.6	31.5 ± 1.6
n-6 series	20.8 ± 2.2	11.0 ± 1.4	12.9 ± 2.9	13.2 ± 1.2	16.8 ± 2.3	15.5 ± 0.9	10.3 ± 0.7	15.0 ± 4.9	17.2 ± 3.9	9.1 ± 1.2
18:2n-6	1.0 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	1.3 ± 0.2	1.4 ± 0.3	1.1 ± 0.1	1.9 ± 0.3	0.8 ± 0.1	0.8 ± 0.1	1.8 ± 0.3
18:3n-6	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
20:2n-6	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
20:3n-6	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.4 ± 0.1
20:4n-6	13.1 ± 1.7	5.3 ± 0.9	7.2 ± 2.1	6.8 ± 0.8	9.5 ± 1.8	8.7 ± 0.7	4.6 ± 0.6	9.2 ± 3.6	11.0 ± 2.9	4.4 ± 1.2
22:4n-6	1.8 ± 0.4	1.4 ± 0.3	1.4 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.1	1.0 ± 0.2	1.5 ± 0.4	1.6 ± 0.3	0.8 ± 0.2
22:5n-6	4.1 ± 0.9	2.1 ± 0.5	2.5 ± 0.7	2.7 ± 0.4	3.6 ± 0.8	3.3 ± 0.3	1.9 ± 0.2	2.8 ± 0.8	3.0 ± 0.8	1.4 ± 0.2
n-3 series	29.2 ± 2.4	13.9 ± 1.1	18.6 ± 2.6	17.5 ± 2.0	21.2 ± 1.7	27.0 ± 1.0	15.9 ± 0.6	24.2 ± 1.1	24.0 ± 2.1	22.4 ± 1.5
18:3n-3	0.2 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.5 ± 0.1
18:4n-3	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.1
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
20:4n-3	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0
20:5n-3	2.5 ± 0.2	1.2 ± 0.1	2.0 ± 0.3	1.7 ± 0.4	2.1 ± 0.1	3.2 ± 0.3	1.3 ± 0.1	4.1 ± 0.4	2.6 ± 0.2	2.3 ± 0.4
22:5n-3	3.5 ± 0.2	3.1 ± 0.3	3.9 ± 0.4	3.9 ± 0.7	3.3 ± 0.6	4.9 ± 0.4	2.6 ± 0.3	5.2 ± 0.7	5.1 ± 0.6	5.3 ± 0.3
22:6n-3	22.4 ± 2.1	8.2 ± 0.9	11.5 ± 2.0	10.8 ± 1.2	15.0 ± 2.0	17.7 ± 0.5	11.0 ± 0.4	13.9 ± 1.7	15.5 ± 1.9	13.4 ± 1.9

[a]Results are expressed as weight percentage of total FA.
[b]Data are mean ± SE.

TABLE 3
FA Composition of *S. fuscescens* Liver[a,b]

	2002												2003
	Apr. 11	Apr. 23	May 25	Jun. 6	Jul. 9	Aug. 1	Aug. 7	Sept. 30	Oct. 16	Nov. 8	Nov. 27	Dec. 19	Apr. 10
Total saturated	41.8 ± 0.7	37.4 ± 1.4	38.6 ± 1.9	41.4 ± 1.0	40.9 ± 1.3	46.3 ± 2.8	51.6 ± 2.6	43.7 ± 2.0	45.0 ± 1.0	38.5 ± 0.4	37.5 ± 1.7	37.1 ± 1.4	35.8 ± 1.2
12:0	0.7 ± 0.2	0.6 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	2.4 ± 1.0	0.6 ± 0.3	0.0 ± 0.0	0.4 ± 0.2	0.5 ± 0.0	0.4 ± 0.1	0.3 ± 0.2	0.1 ± 0.1
14:0	3.5 ± 0.5	2.8 ± 0.6	2.4 ± 0.1	2.7 ± 0.4	2.3 ± 0.7	5.7 ± 1.0	3.4 ± 0.9	2.5 ± 0.4	2.3 ± 0.2	2.2 ± 0.1	2.2 ± 0.2	2.6 ± 0.3	3.2 ± 0.4
15:0	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.3	1.0 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
16:0	30.5 ± 0.6	26.9 ± 1.0	29.0 ± 3.1	29.7 ± 0.7	29.4 ± 1.4	29.8 ± 2.0	36.6 ± 2.2	34.0 ± 1.9	33.4 ± 1.1	27.3 ± 0.4	28.1 ± 0.6	25.6 ± 1.5	25.0 ± 1.2
17:0	0.6 ± 0.2	0.9 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.2 ± 0.1
18:0	5.4 ± 0.4	5.2 ± 0.3	5.3 ± 1.2	7.2 ± 0.1	6.9 ± 0.4	6.1 ± 0.7	8.2 ± 0.6	6.5 ± 0.5	8.1 ± 0.4	7.3 ± 0.3	5.3 ± 0.9	6.6 ± 0.3	4.9 ± 0.5
19:0	0.6 ± 0.2	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
20:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	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	29.0 ± 5.3	18.0 ± 2.8	17.9 ± 3.9	21.0 ± 4.6	13.3 ± 1.3	21.9 ± 1.7	17.2 ± 2.0	24.0 ± 1.9	32.3 ± 0.8	27.7 ± 5.1	33.7 ± 5.1	17.5 ± 2.6	18.0 ± 3.3
16:1n-7	13.9 ± 3.0	6.4 ± 1.5	8.2 ± 3.0	5.9 ± 1.9	4.4 ± 1.1	9.0 ± 0.9	5.9 ± 0.8	9.0 ± 0.9	8.9 ± 1.0	7.6 ± 0.6	13.2 ± 2.0	6.8 ± 1.5	7.1 ± 1.1
18:1n-9	9.8 ± 1.7	8.1 ± 1.7	6.5 ± 1.0	11.1 ± 2.2	5.6 ± 0.2	8.5 ± 1.2	7.5 ± 1.1	10.7 ± 1.6	18.5 ± 0.8	15.1 ± 3.8	12.7 ± 2.3	6.6 ± 0.9	7.0 ± 1.6
18:1n-7	4.8 ± 0.7	2.9 ± 0.7	2.4 ± 0.1	3.1 ± 0.3	3.0 ± 0.3	3.6 ± 0.1	3.4 ± 0.4	3.6 ± 0.1	4.3 ± 0.1	4.0 ± 0.7	6.8 ± 1.0	3.2 ± 0.4	2.9 ± 0.7
18:1n-5	0.0 ± 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	0.4 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:1n-11	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.1 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	0.2 ± 0.0	0.6 ± 0.1
20:1n-9	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.2	0.1 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
22:1n-11	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	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 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	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1n-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	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Total polyenoic	26.1 ± 4.7	38.3 ± 4.4	39.9 ± 5.2	31.5 ± 5.5	40.2 ± 2.7	25.9 ± 1.8	25.3 ± 1.9	26.3 ± 3.5	18.9 ± 1.6	29.4 ± 4.9	25.5 ± 3.4	39.4 ± 3.6	42.5 ± 2.6
n-6 series	9.1 ± 1.0	11.4 ± 1.7	10.3 ± 1.0	10.0 ± 0.1	11.4 ± 0.4	8.1 ± 0.8	10.3 ± 0.8	11.5 ± 1.4	9.2 ± 0.5	10.0 ± 1.2	8.4 ± 1.3	13.5 ± 1.5	12.1 ± 0.9
18:2n-6	0.9 ± 0.2	0.7 ± 0.1	0.7 ± 0.0	1.0 ± 0.1	1.4 ± 0.7	0.8 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	2.0 ± 0.2	1.1 ± 0.2	0.4 ± 0.1	0.7 ± 0.1	1.2 ± 0.4
18:3n-6	0.0 ± 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	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.8 ± 0.2	0.8 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
20:3n-6	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
20:4n-6	6.1 ± 0.9	7.4 ± 1.4	6.6 ± 0.6	5.6 ± 0.1	7.8 ± 0.9	4.5 ± 0.4	6.1 ± 0.4	6.0 ± 0.9	3.6 ± 0.4	5.5 ± 0.9	5.3 ± 1.1	8.4 ± 1.0	7.1 ± 1.0
22:4n-6	0.4 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	0.5 ± 0.0	0.7 ± 0.2	0.6 ± 0.1	1.1 ± 0.2	0.6 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	1.2 ± 0.2	1.1 ± 0.2
22:5n-6	1.5 ± 0.2	1.9 ± 0.3	1.5 ± 0.1	1.8 ± 0.1	1.1 ± 0.2	1.5 ± 0.3	2.5 ± 0.4	2.6 ± 0.5	1.7 ± 0.2	1.7 ± 0.3	1.6 ± 0.2	2.7 ± 0.3	1.9 ± 0.3
n-3 series	17.0 ± 4.2	27.0 ± 3.0	29.6 ± 4.2	21.5 ± 5.6	28.7 ± 2.4	17.8 ± 1.1	15.0 ± 1.5	14.8 ± 2.1	9.7 ± 1.2	19.4 ± 3.8	17.2 ± 2.2	25.9 ± 2.5	30.4 ± 1.9
18:3n-3	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.6 ± 0.2	0.3 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.4 ± 0.1
18:4n-3	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.2	0.2 ± 0.1
20:3n-3	0.0 ± 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	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:4n-3	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.0	0.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.5 ± 0.1	0.7 ± 0.1
20:5n-3	1.4 ± 0.5	2.2 ± 0.5	1.7 ± 0.3	1.2 ± 0.4	3.1 ± 0.4	0.8 ± 0.2	0.8 ± 0.2	0.6 ± 0.1	0.4 ± 0.0	1.1 ± 0.3	1.4 ± 0.3	1.6 ± 0.3	2.9 ± 0.6
22:5n-3	2.0 ± 0.3	4.2 ± 0.6	5.0 ± 1.3	2.5 ± 0.1	3.4 ± 0.2	2.4 ± 0.2	1.7 ± 0.4	2.6 ± 0.4	1.3 ± 0.2	3.0 ± 0.7	2.5 ± 0.3	3.1 ± 0.1	5.0 ± 0.4
22:6n-3	12.9 ± 3.5	19.5 ± 2.1	21.8 ± 2.5	17.0 ± 4.9	20.5 ± 2.3	13.9 ± 1.2	11.8 ± 1.1	11.2 ± 1.7	7.7 ± 0.9	14.2 ± 2.8	12.6 ± 2.0	19.5 ± 2.2	21.0 ± 1.8

[a]Results are expressed as weight percentage of total FA.
[b]Data are mean ± SE.

TABLE 3 (continued)
FA Composition of *S. fuscescens* Liver[a,b]

	2003		2004							
	Aug. 1	Nov. 18	Jan. 9	Feb. 17	Mar. 11	Apr. 21	May 22	Jun. 18	Jul. 30	Sept. 29
Total saturated	45.1 ± 2.7	38.3 ± 1.3	37.1 ± 1.2	39.2 ± 1.0	38.9 ± 1.2	37.3 ± 0.4	40.2 ± 0.4	39.4 ± 1.2	42.8 ± 2.3	36.3 ± 0.7
12:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.6	0.0 ± 0.0
14:0	2.6 ± 0.3	2.1 ± 0.1	3.2 ± 0.3	4.3 ± 0.3	3.1 ± 0.2	3.1 ± 0.2	1.9 ± 0.1	1.7 ± 0.2	4.3 ± 1.2	1.9 ± 0.2
15:0	0.8 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.9 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.3 ± 0.0
16:0	32.0 ± 1.8	25.7 ± 1.5	26.5 ± 1.5	27.4 ± 0.9	27.1 ± 1.3	27.6 ± 0.5	29.8 ± 0.3	29.1 ± 2.4	28.9 ± 0.9	27.8 ± 0.5
17:0	0.4 ± 0.1	0.6 ± 0.3	0.6 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.0	0.6 ± 0.1	0.9 ± 0.2	0.6 ± 0.3	0.5 ± 0.0
18:0	8.6 ± 0.8	9.0 ± 0.3	5.7 ± 0.3	5.7 ± 0.3	6.1 ± 0.4	4.4 ± 0.5	6.9 ± 0.1	6.6 ± 1.3	6.7 ± 0.3	5.4 ± 0.4
19:0	0.4 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.2 ± 0.0
20:0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
Total monoenoic	15.3 ± 1.4	23.5 ± 3.5	19.3 ± 2.4	22.9 ± 3	20.4 ± 2.6	19.8 ± 2	15.2 ± 1.5	17.4 ± 4.8	21.0 ± 4.2	29.6 ± 4.1
16:1n-7	4.6 ± 0.6	5.5 ± 0.9	8.0 ± 1.0	10.2 ± 1.5	8.4 ± 1.6	9.3 ± 1.9	4.3 ± 0.5	6.5 ± 3.0	9.8 ± 2.4	7.5 ± 0.8
18:1n-9	7.1 ± 0.7	12.8 ± 2.2	7.0 ± 1.1	8.9 ± 1.3	8.3 ± 0.7	6.0 ± 0.4	7.8 ± 0.8	7.3 ± 1.1	6.3 ± 1.0	16.0 ± 2.9
18:1n-7	3.1 ± 0.3	4.2 ± 0.4	3.2 ± 0.4	3.3 ± 0.2	3.2 ± 0.3	3.6 ± 0.2	2.6 ± 0.2	2.7 ± 0.4	4.4 ± 0.7	4.8 ± 0.4
18:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
20:1n-11	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.4 ± 0.2	0.2 ± 0.0	0.5 ± 0.1
20:1n-9	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
22:1n-11	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 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	0.1 ± 0.1	0.1 ± 0.0
24:1n-9	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total polyenoic	31.9 ± 4.3	32.0 ± 3.7	36.3 ± 3	32.4 ± 3.8	36.8 ± 3.2	38.3 ± 2.1	40.3 ± 1.7	38.5 ± 5.2	31.6 ± 4.1	29.9 ± 3.6
n-6 series	9.9 ± 0.9	13.5 ± 1.9	12.2 ± 1.1	11.8 ± 1.6	12.7 ± 1.4	9.9 ± 0.4	13.7 ± 1.7	14.1 ± 1.4	8.9 ± 0.7	9.6 ± 1.9
18:2n-6	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	1.3 ± 0.2
18:3n-6	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.2	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
20:2n-6	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
20:3n-6	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1
20:4n-6	6.4 ± 0.7	7.4 ± 1.2	7.7 ± 0.9	6.8 ± 1.0	7.1 ± 0.9	5.8 ± 0.4	9.1 ± 1.5	8.2 ± 1.2	6.0 ± 0.8	4.7 ± 1.4
22:4n-6	0.7 ± 0.0	1.6 ± 0.3	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	0.7 ± 0.0	1.0 ± 0.2	1.1 ± 0.1	0.7 ± 0.1	1.1 ± 0.2
22:5n-6	1.6 ± 0.4	2.9 ± 0.6	2.1 ± 0.2	2.5 ± 0.5	2.8 ± 0.4	1.8 ± 0.1	2.2 ± 0.1	3.7 ± 0.4	1.2 ± 0.3	1.9 ± 0.4
n-3 series	22.0 ± 3.4	18.5 ± 2	24.1 ± 2.7	20.7 ± 2.5	24.1 ± 1.8	28.4 ± 1.7	26.5 ± 0.6	24.4 ± 4.1	22.6 ± 3.4	20.3 ± 1.8
18:3n-3	0.2 ± 0	0.3 ± 0	0.4 ± 0.1	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.1 ± 0	0.2 ± 0	0.2 ± 0
18:4n-3	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n-3	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
20:4n-3	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
20:5n-3	1.1 ± 0.3	0.7 ± 0.1	2.4 ± 0.2	1.6 ± 0.4	2.2 ± 0.4	1.8 ± 0.2	1.7 ± 0.1	1.2 ± 0.2	1.7 ± 0.1	1.0 ± 0.2
22:5n-3	2.1 ± 0.2	2.9 ± 0.3	3.8 ± 0.3	4.0 ± 0.6	4.1 ± 0.4	3.5 ± 0.2	3.4 ± 0.5	2.4 ± 0.1	3.8 ± 0.2	4.4 ± 0.6
22:6n-3	18.1 ± 3.2	13.8 ± 1.8	16.6 ± 2.4	13.9 ± 1.5	16.8 ± 1.4	22.1 ± 1.4	20.6 ± 1.0	20.4 ± 3.8	16.4 ± 3.6	14.3 ± 1.1

[a]Results are expressed as weight percentage of total FA.
[b]Data are mean ± SE.

TABLE 4
FA Composition of *S. fuscescens* Other Viscera[a,b]

	2002												2003
	Apr. 11	Apr. 23	May 25	Jun. 6	Jul. 9	Aug. 1	Aug. 7	Sept. 30	Oct. 16	Nov. 8	Nov. 27	Dec. 19	Apr. 10
Total saturated	44.4 ± 1.2	45.9 ± 2.1	38.1 ± 1.8	41.4 ± 0.5	36.5 ± 1.0	36.9 ± 1.1	40.1 ± 1.2	45.8 ± 1.5	43.6 ± 0.8	43.8 ± 0.4	39.2 ± 2.0	42.6 ± 2.0	40.5 ± 1.8
12:0	1.0 ± 0.1	0.9 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.2
14:0	4.3 ± 0.4	4.8 ± 0.3	5.8 ± 0.2	4.2 ± 0.1	4.0 ± 0.2	3.0 ± 0.4	3.0 ± 0.4	5.5 ± 0.4	4.1 ± 0.2	4.0 ± 0.4	3.8 ± 0.3	4.4 ± 0.3	4.9 ± 0.3
15:0	0.4 ± 0.1	0.6 ± 0.1	1.3 ± 0.2	0.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
16:0	31.4 ± 1.4	30.5 ± 1.6	23.6 ± 1.7	28.1 ± 1.6	24.8 ± 0.5	23.7 ± 0.9	26.7 ± 1.0	34.0 ± 1.7	31.9 ± 0.8	32.0 ± 0.2	27.6 ± 1.4	29.6 ± 2.2	27.8 ± 1.6
17:0	0.4 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.4 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.6 ± 0.1
18:0	5.7 ± 0.2	7.4 ± 1.0	5.9 ± 0.6	7.7 ± 0.9	5.1 ± 0.5	8.2 ± 0.9	8.0 ± 0.3	5.0 ± 0.5	6.0 ± 0.2	5.8 ± 0.3	5.3 ± 0.4	5.8 ± 0.5	5.3 ± 0.3
19:0	0.9 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
20:0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
Total monoenoic	40.5 ± 2.0	32.1 ± 1.9	18.4 ± 1.8	32.4 ± 2.0	21.4 ± 0.7	20.3 ± 2.3	20.7 ± 1.8	31.5 ± 0.7	36.9 ± 1.2	35.9 ± 1.8	37.5 ± 2.7	28.2 ± 3.6	32.1 ± 1.7
16:1n-7	16.6 ± 0.5	11.3 ± 0.8	5.8 ± 0.4	8.2 ± 1.7	8.5 ± 0.7	5.5 ± 1.4	5.7 ± 0.8	15.0 ± 1.0	12.7 ± 0.3	12.5 ± 0.3	14.2 ± 0.8	9.5 ± 2.5	11.7 ± 0.8
18:1n-9	16.9 ± 1.8	15.1 ± 1.1	8.0 ± 1.0	18.3 ± 0.5	8.4 ± 0.3	10.9 ± 0.9	10.8 ± 0.6	12.0 ± 1.3	19.3 ± 1.6	18.1 ± 1.7	15.4 ± 1.0	13.4 ± 1.1	15.1 ± 1.3
18:1n-7	6.0 ± 0.5	4.6 ± 0.8	2.7 ± 0.3	4.0 ± 0.0	3.7 ± 0.3	3.0 ± 0.0	3.4 ± 0.3	3.8 ± 0.1	3.9 ± 0.5	4.1 ± 0.1	6.5 ± 0.9	4.0 ± 0.2	3.7 ± 0.2
18:1n-5	0.4 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.2 ± 0.0	0.1 ± 0.0
20:1n-11	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.9 ± 0.4	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.7 ± 0.1
20:1n-9	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
22:1n-11	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
22:1n-9	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
24:1n-9	0.0 ± 0.0	0.1 ± 0.0	0.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Total polyenoic	12.4 ± 2.3	16.1 ± 2.7	34.9 ± 2.8	16.8 ± 0.6	32.7 ± 0.9	37.5 ± 2.7	33.9 ± 3.2	14.1 ± 1.1	14.7 ± 0.8	14.4 ± 1.3	17.8 ± 1.9	21.2 ± 3.7	21.3 ± 2.6
n-6 series	6.3 ± 0.5	7.7 ± 1.0	10.8 ± 0.2	7.4 ± 0.3	9.2 ± 0.6	13.5 ± 1.2	12.8 ± 1.5	5.1 ± 0.4	5.6 ± 0.5	5.9 ± 0.3	5.8 ± 0.7	8.7 ± 1.5	7.8 ± 0.9
18:2n-6	1.9 ± 0.3	1.7 ± 0.3	1.1 ± 0.0	1.7 ± 0.0	1.9 ± 0.5	0.9 ± 0.1	0.8 ± 0.0	0.7 ± 0.0	1.8 ± 0.4	1.8 ± 0.3	1.5 ± 0.3	1.4 ± 0.1	1.7 ± 0.4
18:3n-6	0.0 ± 0.0	0.4 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:2n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
20:3n-6	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.1	0.4 ± 0.0	0.4 ± 0.0
20:4n-6	2.4 ± 0.4	3.0 ± 0.7	5.3 ± 0.3	2.7 ± 0.3	4.6 ± 0.2	7.5 ± 1.0	7.4 ± 1.1	2.4 ± 0.2	2.0 ± 0.1	2.2 ± 0.2	2.2 ± 0.5	3.5 ± 0.8	2.9 ± 0.5
22:4n-6	0.6 ± 0.1	0.9 ± 0.2	1.8 ± 0.3	0.9 ± 0.1	0.8 ± 0.1	1.8 ± 0.2	1.2 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	1.3 ± 0.3	1.1 ± 0.3
22:5n-6	0.9 ± 0.3	1.1 ± 0.3	1.6 ± 0.1	1.2 ± 0.0	1.0 ± 0.1	2.6 ± 0.3	2.9 ± 0.4	0.7 ± 0.1	0.7 ± 0.0	0.6 ± 0.1	0.9 ± 0.2	1.6 ± 0.4	1.4 ± 0.4
n-3 series	6.1 ± 2.0	8.4 ± 1.8	24.2 ± 3.1	9.4 ± 0.9	23.5 ± 0.9	24.0 ± 1.6	21.1 ± 1.8	9.1 ± 0.7	9.1 ± 0.6	8.5 ± 1.2	12.0 ± 1.9	12.5 ± 2.3	13.5 ± 2.2
18:3n-3	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.2	0.2 ± 0.0	1.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
18:4n-3	0.2 ± 0.0	0.4 ± 0.1	0.9 ± 0.5	0.1 ± 0.0	0.8 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0
20:3n-3	0.0 ± 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	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:4n-3	0.3 ± 0.0	0.3 ± 0.1	0.9 ± 0.2	0.2 ± 0.0	1.0 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.0
20:5n-3	0.7 ± 0.3	1.0 ± 0.4	2.5 ± 0.8	0.6 ± 0.1	3.7 ± 0.4	1.5 ± 0.2	1.6 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	1.1 ± 0.3	1.6 ± 0.4	1.4 ± 0.4	1.5 ± 0.2
22:5n-3	1.7 ± 0.7	2.6 ± 0.6	6.6 ± 0.5	2.0 ± 0.0	5.6 ± 0.2	4.7 ± 0.3	3.5 ± 0.2	2.4 ± 0.2	1.8 ± 0.2	2.1 ± 0.3	2.6 ± 0.4	3.4 ± 0.7	3.6 ± 0.4
22:6n-3	2.9 ± 0.9	3.7 ± 0.7	12.6 ± 1.9	6.1 ± 0.7	11.0 ± 0.6	16.9 ± 1.4	15.0 ± 1.6	4.3 ± 0.4	5.1 ± 0.3	3.7 ± 0.5	6.4 ± 1.6	5.8 ± 1.1	7.1 ± 1.6

[a]Results are expressed as weight percentage of total FA.
[b]Data are mean ± SE.

TABLE 4 (continued)
FA Composition of *S. fuscescens* Other Viscera[a,b]

	2003		2004							
	Aug. 1	Nov. 18	Jan. 9	Feb. 17	Mar. 11	Apr. 21	May 22	Jun. 18	Jul. 30	Sept. 29
Total saturated	34.7 ± 1.1	37.5 ± 1.0	42.4 ± 1.8	42.4 ± 1.5	43.8 ± 1.1	41.1 ± 1.1	42.5 ± 0.2	38.9 ± 0.5	38.2 ± 0.8	39.1 ± 1.4
12:0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	0.0 ± 0.0	0.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0
14:0	2.4 ± 0.3	3.8 ± 0.1	4.5 ± 0.3	4.6 ± 0.3	4.3 ± 0.2	5.8 ± 0.4	3.9 ± 0.1	3.5 ± 0.8	2.9 ± 0.5	4.9 ± 0.5
15:0	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.5 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
16:0	22.9 ± 0.3	26.1 ± 1.2	29.0 ± 2.3	29.0 ± 1.7	29.4 ± 1.1	27.4 ± 1.0	30.2 ± 0.4	25.3 ± 1.7	25.6 ± 0.5	26.8 ± 1.0
17:0	0.5 ± 0.0	0.7 ± 0.3	0.7 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
18:0	7.8 ± 1.1	5.8 ± 0.6	6.5 ± 0.4	6.6 ± 0.4	7.5 ± 0.6	5.7 ± 0.8	7.1 ± 0.5	7.8 ± 2.0	7.5 ± 0.3	5.2 ± 0.6
19:0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.1
20:0	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.1
Total monoenoic	19.5 ± 0.8	33.1 ± 3.6	29.7 ± 3.0	30.0 ± 3.4	33.5 ± 2.3	26.5 ± 2.2	35.6 ± 1.2	20.7 ± 3.6	18.7 ± 2.4	29.2 ± 1.4
16:1n-7	4.5 ± 0.9	10.8 ± 2.0	10.9 ± 0.9	10.3 ± 1.1	11.3 ± 1.1	9.5 ± 0.3	10.0 ± 0.6	8.0 ± 3.0	5.9 ± 1.9	9.1 ± 1.4
18:1n-9	10.7 ± 0.5	16.7 ± 1.8	13.6 ± 2.2	14.6 ± 2.4	16.9 ± 1.3	11.5 ± 2.3	20.2 ± 0.6	8.5 ± 0.5	8.3 ± 0.2	13.8 ± 1.4
18:1n-7	3.2 ± 0.1	4.5 ± 0.2	3.8 ± 0.3	3.7 ± 0.2	3.9 ± 0.1	4.0 ± 0.1	4.1 ± 0.1	3.0 ± 0.2	3.8 ± 0.4	3.7 ± 0.2
≤18:1n-5	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
20:1n-11	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.2	0.1 ± 0.1	1.0 ± 0.2
20:1n-9	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.4 ± 0.1
22:1n-11	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2
22:1n-9	0.0 ± 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	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
24:1n-9	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.1
Total polyenoic	35.3 ± 1.8	19.1 ± 2.8	20.4 ± 3.5	21.6 ± 4.1	15.8 ± 2.1	25.8 ± 3.5	14.5 ± 1.1	32.6 ± 2.8	38.2 ± 3.7	25.7 ± 0.6
n-6 series	13.0 ± 0.4	8.5 ± 1.6	8.4 ± 1.4	9.0 ± 1.1	7.9 ± 1.0	8.1 ± 0.6	7.4 ± 0.4	12.3 ± 2.9	16.1 ± 2.3	7.2 ± 0.6
18:2n-6	1.0 ± 0.1	1.2 ± 0.3	1.5 ± 0.2	1.5 ± 0.3	2.0 ± 0.4	1.8 ± 0.2	2.5 ± 0.4	0.6 ± 0.1	0.7 ± 0.0	2.2 ± 0.4
18:3n-6	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
20:2n-6	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
20:3n-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.5 ± 0.2	0.4 ± 0.1
20:4n-6	7.0 ± 0.3	3.8 ± 0.9	3.3 ± 0.7	3.5 ± 0.8	2.3 ± 0.6	3.3 ± 0.5	2.2 ± 0.5	7.0 ± 2.0	10.7 ± 1.8	2.7 ± 0.7
22:4n-6	1.3 ± 0.0	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	0.8 ± 0.0	0.8 ± 0.2	1.3 ± 0.3	1.7 ± 0.3	0.8 ± 0.2
22:5n-6	2.6 ± 0.3	1.4 ± 0.5	1.4 ± 0.4	1.7 ± 0.4	1.3 ± 0.4	1.1 ± 0.1	0.9 ± 0.1	2.7 ± 0.6	2.3 ± 0.4	0.8 ± 0.1
n-3 series	22.3 ± 1.9	10.6 ± 1.4	12.1 ± 2.2	12.6 ± 3.1	8.0 ± 1.1	17.6 ± 3.0	7.1 ± 0.7	20.3 ± 0.5	22.1 ± 2.3	18.5 ± 1.0
18:3n-3	0.3 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.2
18:4n-3	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.1
20:3n-3	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
20:4n-3	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.6 ± 0.0
20:5n-3	0.9 ± 0.2	1.1 ± 0.1	1.3 ± 0.3	1.4 ± 0.5	0.7 ± 0.2	2.5 ± 0.6	0.5 ± 0.1	2.9 ± 0.8	2.2 ± 0.1	2.4 ± 0.4
22:5n-3	3.1 ± 0.4	2.8 ± 0.4	3.4 ± 0.7	3.9 ± 1.0	2.7 ± 0.4	3.9 ± 0.6	1.6 ± 0.3	4.3 ± 0.7	5.3 ± 0.3	4.8 ± 0.4
22:6n-3	17.1 ± 1.9	5.0 ± 1.0	5.8 ± 1.1	6.1 ± 1.6	3.6 ± 0.5	9.5 ± 1.7	4.2 ± 0.2	12.1 ± 1.3	14.0 ± 2.1	9.4 ± 1.7

[a]Results are expressed as weight percentage of total FA.
[b]Data are mean ± SE.

TABLE 5
FA Composition of *S. fuscescens* Stomach Contents[a,b]

	2002												2003
	Apr. 11	Apr. 23	May 25	Jun. 6	Jul. 9	Aug. 1	Aug. 7	Sept. 30	Oct. 16	Nov. 8	Nov. 27	Dec. 19	Apr. 10
Total saturated	37.6 ± 0.5	36.8 ± 0.2	23.9 ± 7.1	35.6 ± 1.5	35.1 ± 0.8	27.6 ± 5.7	37.5 ± 2.6	32.3 ± 2.2	40.3 ± 3.4	35.1 ± 0.8	38.0 ± 1.8	37.3 ± 0.9	34.0 ± 0.4
12:0	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
14:0	1.7 ± 0.1	1.5 ± 0.3	1.2 ± 0.9	2.4 ± 0.5	3.1 ± 0.0	0.5 ± 0.2	2.0 ± 0.2	2.2 ± 0.3	3.1 ± 0.9	1.8 ± 0.1	1.9 ± 0.4	2.2 ± 0.4	2.8 ± 0.2
15:0	0.7 ± 0.1	0.7 ± 0.1	0.4 ± 0.3	1.0 ± 0.2	0.7 ± 0.1	1.4 ± 0.6	1.0 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	0.8 ± 0.1
16:0	22.8 ± 0.4	21.3 ± 0.5	14.4 ± 3.6	19.6 ± 1.0	20.3 ± 0.5	12.5 ± 3.4	19.4 ± 2.1	18.3 ± 1.1	27.2 ± 3.5	21.2 ± 0.9	22.9 ± 1.9	22.1 ± 1.3	20.5 ± 1.0
17:0	0.9 ± 0.2	1.0 ± 0.1	0.5 ± 0.4	1.0 ± 0.0	0.8 ± 0.1	0.8 ± 0.3	0.9 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	0.6 ± 0.2
18:0	10.5 ± 0.3	11.4 ± 0.4	6.6 ± 1.9	11.1 ± 0.1	9.2 ± 0.5	12.2 ± 2.3	13.3 ± 0.2	10.7 ± 1.5	7.8 ± 1.2	9.8 ± 0.4	10.6 ± 0.8	10.4 ± 1.2	8.4 ± 0.7
19:0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.2 ± 0.2	0.7 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
20:0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Total monoenoic	16.7 ± 2.4	13.6 ± 0.9	24.0 ± 4.7	14.8 ± 1.0	16.3 ± 0.6	13.2 ± 3.7	10.7 ± 0.4	12.1 ± 0.7	22.2 ± 5.1	14.7 ± 1.1	16.7 ± 1.9	22.2 ± 3.0	17.3 ± 2.8
16:1n-7	5.0 ± 0.9	3.6 ± 0.4	12.4 ± 5.4	3.4 ± 0.6	6.0 ± 0.6	5.1 ± 2.6	2.6 ± 0.1	3.1 ± 0.5	8.1 ± 2.6	4.5 ± 0.5	4.5 ± 1.3	7.2 ± 1.6	5.2 ± 0.8
18:1n-9	7.0 ± 1.3	5.4 ± 0.4	5.0 ± 1.9	7.5 ± 0.4	6.2 ± 0.2	4.2 ± 0.4	4.6 ± 0.3	5.1 ± 0.5	9.8 ± 2.3	6.1 ± 0.4	7.0 ± 0.7	9.0 ± 1.4	6.9 ± 1.8
18:1n-7	4.0 ± 0.2	3.9 ± 0.2	6.1 ± 1.6	3.1 ± 0.0	3.3 ± 0.2	2.8 ± 0.4	3.2 ± 0.2	2.8 ± 0.1	3.4 ± 0.2	3.3 ± 0.3	4.1 ± 0.3	4.8 ± 0.3	3.8 ± 0.1
18:1n-5	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0
20:1n-11	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.6 ± 0.1
20:1n-9	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.5 ± 0.0	0.3 ± 0.0	0.9 ± 0.6	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0
22:1n-11	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
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	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.2
24:1n-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	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
Total polyenoic	40.4 ± 2.8	44.7 ± 1.3	45.4 ± 2.1	39.2 ± 1.6	40.5 ± 0.6	53.5 ± 2.7	43.1 ± 2.2	45.0 ± 1.6	31.7 ± 8.7	41.6 ± 1.8	38.7 ± 3.5	34.6 ± 3.0	40.3 ± 2.5
n-6 series	16.3 ± 1.5	17.5 ± 1.5	14.7 ± 0.9	15.8 ± 1.5	14.2 ± 0.4	33.2 ± 5.7	19.1 ± 1.2	21.9 ± 1.0	14.4 ± 5.1	20.5 ± 0.5	19.7 ± 2.6	12.5 ± 1.5	14.1 ± 1.6
18:2n-6	0.8 ± 0.0	0.8 ± 0.1	1.4 ± 0.0	1.0 ± 0.1	1.9 ± 0.5	0.6 ± 0.3	1.1 ± 0.0	2.0 ± 0.2	1.8 ± 0.5	1.6 ± 0.4	2.0 ± 0.3	1.0 ± 0.1	1.3 ± 0.4
18:3n-6	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 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
20:2n-6	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
20:3n-6	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.5 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	1.0 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
20:4n-6	9.8 ± 0.9	11.8 ± 1.4	9.2 ± 0.4	10.0 ± 1.0	8.6 ± 0.4	26.7 ± 8.4	12.0 ± 0.8	14.1 ± 1.0	8.8 ± 3.5	13.1 ± 0.6	12.8 ± 1.6	7.6 ± 1.1	8.1 ± 1.2
22:4n-6	1.6 ± 0.1	1.3 ± 0.1	1.2 ± 0.3	2.8 ± 0.3	1.2 ± 0.1	1.6 ± 0.7	1.3 ± 0.1	1.8 ± 0.2	1.0 ± 0.2	1.7 ± 0.2	1.3 ± 0.1	1.0 ± 0.1	1.4 ± 0.3
22:5n-6	3.1 ± 0.7	2.7 ± 0.2	2.1 ± 0.5	1.2 ± 0.8	1.7 ± 0.1	3.8 ± 1.6	4.2 ± 0.2	2.7 ± 0.3	1.8 ± 0.6	3.3 ± 0.2	2.7 ± 0.7	2.2 ± 0.3	2.5 ± 0.6
n-3 series	24.1 ± 2.8	27.2 ± 1.0	30.7 ± 3.0	23.4 ± 3.0	26.4 ± 0.8	20.3 ± 4.0	24.0 ± 1.4	23.1 ± 0.9	17.3 ± 3.7	21.1 ± 2.1	19.0 ± 1.0	22.1 ± 2.1	26.2 ± 1.4
18:3n-3	0.3 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	1.1 ± 0.2	0.4 ± 0.3	0.6 ± 0.1	2.0 ± 0.4	1.8 ± 0.5	2.4 ± 1.8	2.0 ± 0.4	0.3 ± 0.1	0.4 ± 0.1
18:4n-3	0.2 ± 0.0	0.2 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	1.0 ± 0.1	1.5 ± 1.1	0.2 ± 0.0	1.0 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.1 ± 0.0	0.3 ± 0.1
20:3n-3	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.9 ± 0.1	0.2 ± 0.1	0.4 ± 0.0	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
20:5n-3	3.1 ± 0.6	3.1 ± 0.4	4.0 ± 0.4	2.4 ± 0.5	5.9 ± 0.5	3.3 ± 1.7	2.4 ± 0.1	3.8 ± 0.3	2.8 ± 0.9	2.3 ± 0.3	2.4 ± 0.3	3.4 ± 0.5	4.6 ± 0.5
22:5n-3	3.1 ± 0.5	4.2 ± 0.3	5.0 ± 0.7	3.1 ± 0.1	5.2 ± 0.1	1.7 ± 0.8	2.5 ± 0.2	2.9 ± 0.3	2.6 ± 0.3	2.6 ± 0.2	1.9 ± 0.5	3.0 ± 0.1	4.5 ± 0.4
22:6n-3	16.9 ± 2.0	19.0 ± 1.3	20.3 ± 2.1	16.6 ± 2.3	12.2 ± 0.5	13.1 ± 5.4	17.8 ± 1.2	12.4 ± 0.7	8.8 ± 1.7	12.5 ± 1.4	11.5 ± 1.3	14.8 ± 2.1	15.7 ± 1.2

[a]Results are expressed as weight percentage of total FA.
[b]Data are mean Å} SE.

TABLE 5 (continued)
FA Composition of *S. fuscescens* Stomach Contents[a,b]

	2003		2004							
	Aug. 1	Nov. 18	Jan. 9	Feb. 17	Mar. 11	Apr. 21	May 22	Jun. 18	Jul. 30	Sept. 29
Total saturated	34.7 ± 1.7	35.5 ± 0.8	36.5 ± 0.8	36.5 ± 1.0	35.9 ± 0.4	35.7 ± 0.7	35.9 ± 1.3	41.4 ± 4.2	35.6 ± 1.1	38.3 ± 1.1
12:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.4 ± 0.2
14:0	0.7 ± 0.1	2.0 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	1.8 ± 0.2	2.4 ± 0.1	2.0 ± 0.2	2.7 ± 0.1	1.3 ± 0.1	2.6 ± 0.5
15:0	0.5 ± 0.0	0.8 ± 0.1	0.4 ± 0.0	0.9 ± 0.2	0.8 ± 0.0	1.2 ± 0.1	0.8 ± 0.1	1.0 ± 0.0	0.7 ± 0.1	0.7 ± 0.0
16:0	17.4 ± 1.3	20.0 ± 1.6	21.4 ± 0.7	21.1 ± 0.9	21.4 ± 0.5	20.8 ± 0.7	21.0 ± 1.5	23.4 ± 3.1	18.5 ± 1.1	22.0 ± 1.1
17:0	0.7 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
18:0	14.8 ± 0.4	11.0 ± 0.8	11.6 ± 0.4	11.9 ± 0.6	10.2 ± 0.2	9.2 ± 0.3	10.6 ± 0.3	12.1 ± 1.0	13.2 ± 0.4	11.2 ± 0.7
19:0	0.4 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
20:0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.1	0.2 ± 0.0
Total monoenoic	7.4 ± 0.9	17.7 ± 2.5	11.8 ± 0.7	12.6 ± 0.8	14.8 ± 1.0	13.9 ± 1.9	14.6 ± 1.2	11.7 ± 0.4	9.6 ± 0.4	17.0 ± 1.5
16:1n-7	1.4 ± 0.3	4.1 ± 0.9	3.3 ± 0.4	3.0 ± 0.3	4.2 ± 0.5	3.9 ± 0.6	3.2 ± 0.3	3.1 ± 0.6	2.3 ± 0.2	4.7 ± 0.8
18:1n-9	3.5 ± 0.5	9.3 ± 1.5	4.6 ± 0.4	6.3 ± 0.5	6.6 ± 0.6	5.4 ± 1.3	7.7 ± 0.8	4.9 ± 0.2	4.0 ± 0.3	8.0 ± 0.9
18:1n-7	2.0 ± 0.1	3.1 ± 0.2	3.2 ± 0.4	2.8 ± 0.2	3.0 ± 0.7	3.5 ± 0.1	3.0 ± 0.2	2.4 ± 0.1	3.1 ± 0.0	3.1 ± 0.1
18:1n-5	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
20:1n-11	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.1 ± 0.0	0.4 ± 0.1
20:1n-9	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.3 ± 0.0
22:1n-11	0.1 ± 0.1	0.6 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1
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
24:1n-9	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	0.2 ± 0.2	0.0 ± 0.0	0.2 ± 0.1
Total polyenoic	54.2 ± 2.5	39.2 ± 3.2	46.0 ± 1.3	45.3 ± 1.3	42.5 ± 1.8	44.8 ± 2.3	40.4 ± 1.7	40.6 ± 3.4	49.2 ± 0.5	37.2 ± 2.9
n-6 series	27.9 ± 3.0	20.9 ± 2.4	19.2 ± 1.5	21.2 ± 1.1	16.0 ± 1.1	14.3 ± 0.6	17.1 ± 2.0	17.6 ± 1.2	23.8 ± 1.5	13.9 ± 1.5
18:2n-6	1.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.5 ± 0.1	0.9 ± 0.1	0.5 ± 0.0	0.9 ± 0.0	2.4 ± 0.4
18:3n-6	0.0 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
20:2n-6	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.3	0.3 ± 0.0
20:3n-6	0.8 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.3 ± 0.1	0.7 ± 0.3	0.4 ± 0.2	0.6 ± 0.1
20:4n-6	18.1 ± 1.7	12.6 ± 1.6	12.7 ± 1.2	11.9 ± 0.4	8.5 ± 0.6	8.4 ± 0.4	10.9 ± 1.8	9.7 ± 1.0	16.3 ± 0.9	7.6 ± 1.5
22:4n-6	2.9 ± 0.6	2.4 ± 0.2	1.5 ± 0.2	2.6 ± 0.3	1.7 ± 0.2	1.1 ± 0.1	1.7 ± 0.3	1.6 ± 0.0	2.3 ± 0.3	1.2 ± 0.2
22:5n-6	4.5 ± 0.8	3.9 ± 0.7	2.9 ± 0.3	4.7 ± 0.5	4.1 ± 0.5	2.4 ± 0.1	2.9 ± 0.1	4.6 ± 0.5	3.5 ± 0.7	1.8 ± 0.2
n-3 series	26.3 ± 0.6	18.3 ± 1.2	26.8 ± 2.1	24.2 ± 0.5	26.5 ± 2.0	30.4 ± 1.9	23.3 ± 0.4	23.0 ± 2.3	25.4 ± 1.6	23.3 ± 2.5
18:3n-3	0.5 ± 0.1	1.2 ± 0.2	0.4 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.1
18:4n-3	0.2 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1
20:3n-3	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	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
20:4n-3	0.7 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.8 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.4 ± 0.1
20:5n-3	1.2 ± 0.2	1.7 ± 0.2	3.6 ± 0.3	2.8 ± 0.3	4.3 ± 0.4	5.0 ± 0.5	3.1 ± 0.3	3.6 ± 0.9	3.3 ± 0.2	3.0 ± 0.4
22:5n-3	3.3 ± 0.3	2.6 ± 0.1	3.3 ± 0.4	4.5 ± 0.5	3.8 ± 0.2	4.3 ± 0.2	2.9 ± 0.3	3.1 ± 0.6	4.4 ± 0.4	4.0 ± 0.7
22:6n-3	20.1 ± 0.5	11.4 ± 1.1	18.4 ± 2.2	15.8 ± 0.8	17.0 ± 1.6	19.5 ± 1.2	16.1 ± 1.0	15.6 ± 0.7	17.2 ± 1.4	14.9 ± 2.9

[a]Results are expressed as weight percentage of total FA.
[b]Data are mean ± SE.

TABLE 6
NL and PL in Muscle, Liver, and Other Viscera of *S. fuscescens*[a,b]

		Muscle	Liver	Other viscera
Fatty[c] (*n* = 10)	NL[d]	1,407.4 (80.0 ± 137.8)[e]	8,607.9 (68.2) ± 2,888.9	32,127.5 (93.6) ± 3,948.3
	FFA	46.6 (2.6) ± 6.0	2,400.1 (19.0) ± 388.6	1,903.1 (5.5) ± 184.4
	PL[f]	305.7 (17.4) ± 46.6	1,611.0 (12.8) ± 420.6	291.9 (0.9) ± 76.0
Lean[g] (*n* = 11)	NL[d]	527.7 (66.8) ± 158.7	1,301.1 (35.1) ± 361.1	2,491.4 (67.5) ± 631.7
	FFA	43.7 (5.5) ± 3.3	1,384.1 (37.4) ± 226.0	789.3 (21.4) ± 177.9
	PL[f]	218.9 (27.7) ± 23.0	1,019.5 (27.5) ± 111.6	410.3 (11.1) ± 45.8

[a]Results are expressed as contents (mg) in 100 g of respective tissues.
[b]Data are mean ± SE.
[c]Fatty indicates the fish samples collected on November 27, 2002, and November 18, 2003.
[d]There are significant differences ($P < 0.05$) in the actual NL values (mg/100 g) of each organ between fatty fish and lean fish.
[e]Numerals in parenthesis are weight percentage of total lipids.
[f]There are no significant differences ($P > 0.05$) in the actual PL values (mg/100 g) of each organ between fatty fish and lean fish.
[g]Lean indicates fish collected on July 9, 2002, August 1, 2003, and July 30, 2004.

year (17.5–33.7%). Characteristic seasonal changes could not be found, however, in the FA compositions of other viscera and stomach contents. In the other viscera TL, the total SFA levels were almost constant throughout the year at 40.8 ± 0.6%. Total MUFA levels fluctuated widely (18.4–40.5%), with lower values (18.4–21.4%) obtained on May 25, July 9, August 1, and August 7, 2002; August 1, 2003; and June 18 and July 30, 2004, compared with other samples (26.5–40.5%). In the stomach contents, total SFA levels were almost constant at 35.5 ± 0.8%, except for lower values observed in samples obtained on May 25 (23.9 ± 7.1%) and August 1, 2002 (27.6 ± 5.7%). Similar to MUFA of other viscera, total MUFA levels in stomach contents fluctuated widely (7.4–24.0%), with lower values observed on August 7, 2002 (10.7 ± 0.4%); August 1, 2003 (7.4 ± 0.9%); and July 30, 2004 (9.6 ± 0.4%).

MUFA levels in TL of muscle and liver varied in accordance with their TL levels, indicating that seasonal variations in TL were largely due to those of MUFA. No seasonal changes existed in the MUFA levels of stomach content lipids, as already mentioned. Moreover, the annual average total MUFA level of stomach contents (15.0 ± 0.8%) was significantly lower than those of muscle (24.0 ± 1.1%) or liver (21.6 ± 1.1%). These findings suggest that *S. fuscescens* seasonally stores MUFA selectively in muscle and liver as depot fats.

In TL of muscle and liver, the AA levels varied in accordance with total PUFA levels, and relatively high values of AA were observed between late May and August (muscle, 4.6–13.1%; liver, 4.5–9.1%) compared with other times of the year (muscle, 4.3–9.5%; liver, 3.6–8.4%). The AA levels in TL of other viscera fluctuated year-round (2.0–10.7%), with higher values in the samples obtained on July 9, August 1, and August 7, 2002; August 1, 2003; and June 18 and July 30, 2004 (7.0–10.7%). Similar to the situation in other viscera, the AA levels in stomach contents on August 1, 2002; August 1, 2003; and July 30, 2004 showed higher values (16.3–26.7%) compared with other samples (7.6–14.1%). This indicates that visceral FA compositions are directly influenced by those of lipids derived from food sources.

The FA composition of NL and PL of S. fuscescens *tissues*. To compare characteristics of the lipids of fatty fish (obtained on November 27, 2002, and November 18, 2003) and lean fish (obtained on July 9, 2002; August 1, 2003; and July 30, 2004), the NL and PL contents and their FA compositions were analyzed (Tables 6–9). In the NL, steryl esters, triacylglycerols, diacylglycerols, and sterols were detected. Phosphatidylethanolamine and phosphatidylcholine were observed in the PL. The contents (mg/100 g) of NL from each organ differed between fatty fish (muscle, 1,407 mg/100 g; liver, 8,608 mg/100 g; other viscera, 32,130 mg/100 g) and lean fish (muscle, 527.7 mg/100 g; liver, 1,301 mg/100 g; other viscera, 2,491 mg/100 g). However, the values for PL were similar between fatty fish (muscle, 305.7 mg/100 g; liver, 1,011 mg/100 g; other viscera, 291.9 mg/100 g) and lean fish (muscle, 218.9 mg/100 g; liver, 1,020 mg/100 g; other viscera, 410.3 mg/100 g). This indicates that the seasonal variations in the TL content were influenced by changes in NL.

The FA composition in NL were similar between organs; the major components were 16:0 (muscle, 24.4–30.1%; liver, 25.9–27.8%; other viscera, 21.6–30.4%), 18:0 (muscle, 4.5–5.3%; liver, 4.8–6.4%; other viscera, 4.7–5.9%), 16:1n-7 (muscle, 10.9–11.7%; liver, 8.6–10.2%; other viscera, 8.9–10.9%), 18:1n-9 (muscle, 10.9–11.7%; liver, 8.6–10.2%; other viscera, 8.9–10.9%), and 22:6n-3 (muscle, 7.0–8.9%; liver, 8.1–13.9%; other viscera, 5.5–12.1%). Lower relative levels of other FA, such as 20:4n-6 (muscle, 2.6–3.8%; liver, 3.8–7.0%; other viscera, 2.6–7.6%) and the n-3 PUFA 20:5n-3 (muscle, 1.4–2.6%; liver, 0.8–2.8%; other viscera, 1.2–3.1%) and 22:5n-3 (muscle, 2.7–5.8%; liver, 2.8–4.5%; other viscera, 2.5–6.3%) were detected.

The major components in PL were 16:0 (muscle, 8.4–15.1%; liver, 17.8–20.3%; other viscera, 17.0–17.4%), 18:0 (muscle, 16.4–19.3%; liver, 13.9–14.0%; other viscera, 11.4–13.7%), 18:1n-9 (muscle, 3.7–5.6%; liver, 3.1–4.5%; other viscera, 4.4–8.2%), the n-6 PUFA 20:4n-6 (muscle, 13.1–14.2%; liver, 12.0–13.6%; other viscera, 7.2–11.9%), and the n-3 PUFA 22:6n-3 (muscle, 26.8–30.5%; liver, 26.6–31.1%; other viscera, 13.2–26.0%). Lower levels of the

TABLE 7
FA Composition of NL in Muscle, Liver, and Other Viscera of *S. fuscescens*[a]

	Fatty			Lean		
	Muscle	Liver	Other Viscera	Muscle	Liver	Other Viscera
Total saturated	40.8 ± 1.3	39.0 ± 1.3	41.9 ± 1.2	36.8 ± 0.5	38.6 ± 2.3	32.9 ± 2.2
12:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
14:0	3.8 ± 0.1	3.3 ± 0.3	3.9 ± 0.2	4.9 ± 0.2	5.2 ± 0.7	4.4 ± 0.3
15:0	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
16:0	30.1 ± 1.4	27.8 ± 1.3	30.4 ± 1.3	24.4 ± 0.6	25.9 ± 2.0	21.6 ± 1.7
17:0	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
18:0	5.3 ± 0.2	6.4 ± 0.7	5.9 ± 0.3	4.5 ± 0.3	4.8 ± 0.2	4.7 ± 0.2
19:0	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	0.5 ± 0.1
20:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
Total monoenoic	33.8 ± 1.8	33.7 ± 2.3	33.7 ± 1.8	25.3 ± 1.6	18.9 ± 1.5	22.7 ± 1.8
16:1n-7	11.7 ± 0.8	10.2 ± 0.9	10.9 ± 0.8	10.9 ± 1.0	8.6 ± 1.0	8.9 ± 0.9
18:1n-9	16.5 ± 1.2	16.7 ± 1.3	17.0 ± 1.2	9.3 ± 0.7	6.7 ± 0.5	9.5 ± 0.9
18:1n-7	4.4 ± 0.2	5.4 ± 0.3	4.4 ± 0.2	3.4 ± 0.3	3.0 ± 0.2	3.2 ± 0.3
18:1n-5	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
20:1n-11	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
20:1n-9	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
22:1n-11	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.5 ± 0.3	0.1 ± 0.1	0.2 ± 0.1
22:1n-9	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1n-9	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Total polyenoic	18.6 ± 2.3	20.2 ± 1.9	16.8 ± 2.0	28.8 ± 2.0	34.6 ± 3.9	37.5 ± 3.6
n-6 series	6.2 ± 0.7	7.6 ± 1.0	6.1 ± 0.9	8.7 ± 0.8	11.5 ± 1.2	13.5 ± 2.1
18:2n-6	1.1 ± 0.2	0.7 ± 0.1	1.1 ± 0.2	1.4 ± 0.3	1.2 ± 0.4	1.5 ± 0.3
18:3n-6	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:2n-6	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
20:3n-6	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.6 ± 0.1
20:4n-6[b]	2.6 ± 0.4 D	3.8 ± 0.6 D	2.6 ± 0.5 D	3.8 ± 0.5 D	7.0 ± 1.1 C	7.6 ± 1.6 C
22:4n-6	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	1.2 ± 0.2	1.0 ± 0.1	1.6 ± 0.3
22:5n-6	1.1 ± 0.2	1.5 ± 0.2	1.1 ± 0.3	1.3 ± 0.2	1.5 ± 0.2	1.9 ± 0.4
n-3 series	12.3 ± 1.9	12.6 ± 1.2	10.7 ± 1.6	20.1 ± 1.8	23.0 ± 3.0	24.0 ± 2.1
18:3n-3	0.5 ± 0.1	0.3 ± 0.0	0.6 ± 0.1	1.0 ± 0.3	0.6 ± 0.1	0.9 ± 0.2
18:4n-3	0.3 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.8 ± 0.2	0.4 ± 0.1	0.6 ± 0.1
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
20:4n-3	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.9 ± 0.2	0.6 ± 0.1	0.9 ± 0.1
20:5n-3	1.4 ± 0.3	0.8 ± 0.2	1.2 ± 0.3	2.6 ± 0.5	2.8 ± 0.5	3.1 ± 0.4
22:5n-3	2.7 ± 0.3	2.8 ± 0.4	2.5 ± 0.3	5.8 ± 0.5	4.5 ± 0.4	6.3 ± 0.6
22:6n-3	7.0 ± 1.5	8.1 ± 0.9	5.5 ± 1.1	8.9 ± 0.8	13.9 ± 2.3	12.1 ± 1.4

[a]Results are expressed as weight percentage of total FA. Data are mean ± SE.
[b]Different letters in the lines of arachidonic acid in Tables 7 and 8 indicate statistical differences ($P < 0.05$).

n-6 PUFA 22:5n-6 (muscle, 4.4%; liver, 2.9–5.4%; other viscera, 3.6–4.3%) and the n-3 PUFA 20:5n-3 (muscle, 1.6–2.2%; liver, 1.0–1.4%; other viscera, 0.9–2.1%) and 22:5n-3 (muscle, 4.2–6.3%; liver, 2.8–3.3%; other viscera, 2.6–6.0%) were detected.

PL contained higher levels of AA than NL throughout the year, indicating that *S. fuscescens* selectively accumulate AA in their PL, as herbivorous fishes do (22).

In both muscle NL and PL, there were no significant differences in AA levels between fatty fish (NL, 2.6 ± 0.4%; PL, 14.2 ± 0.9%) and lean fish (NL, 3.8 ± 0.5%; PL, 13.1 ± 0.6%), indicating that the seasonal difference of AA levels in muscle TL might depend only on the variations in PL. Comparatively high levels of AA and PL with low levels of TL were observed in summer, and lower levels of AA in TL were found with higher levels of TL in winter. This also indicates that AA in NL is utilized with other FA at the same level when the fish is starved, whereas AA in PL is maintained at comparatively high levels regardless of the fish's condition.

In liver, the AA levels in NL of the lean fish (7.0 ± 1.1%) were significantly higher than those in fatty fish (3.8 ± 0.6%), indicating that the seasonal variations in AA levels observed in liver TL depend on the changes of AA levels in NL. PL in contrast contains comparatively high and constant levels of AA. The absolute values of AA in liver NL of lean fish and fatty fish were 74.2 ± 12.6 mg/100 g and 236 ± 65.1 mg/100 g, respectively, indicating that 69% of AA was consumed during the period from winter to summer. However, the liver NL contents decreased 85% from 8,608 ± 2,889 mg/100 g to 1,301 ± 361.1 mg/100 g during the same period. In the PL, there were no significant differences in AA levels (fatty fish, 13.6 ± 1.0%; lean fish, 12.0 ± 0.8%) and actual values (fatty fish, 238.7 ± 69.2 mg/100 g; lean fish, 120.9 ± 17.4 mg/100 g) between fatty and lean fish. This suggests that AA in the liver PL were maintained

TABLE 8
FA Composition of PL in Muscle, Liver, and Other Viscera of *S. fuscescens*[a]

	Fatty			Lean		
	Muscle	Liver	Other Viscera	Muscle	Liver	Other Viscera
Total saturated	32.7 ± 0.9	36.2 ± 1.3	33.4 ± 1.4	29.2 ± 0.5	34.0 ± 1.5	33.2 ± 1.2
12:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
14:0	0.3 ± 0.0	0.3 ± 0.0	2.3 ± 0.2	0.2 ± 0.0	0.4 ± 0.1	0.8 ± 0.1
15:0	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.1
16:0	15.1 ± 1.2	20.2 ± 1.1	17.4 ± 1.0	8.4 ± 0.6	17.8 ± 0.9	17.0 ± 1.0
17:0	0.3 ± 0.1	1.1 ± 0.2	1.0 ± 0.2	0.3 ± 0.0	1.3 ± 0.3	0.6 ± 0.1
18:0	16.4 ± 0.8	14.0 ± 1.3	11.4 ± 0.6	19.3 ± 0.6	13.9 ± 0.7	13.7 ± 0.6
19:0	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
20:0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
Total monoenoic	9.5 ± 0.6	8.0 ± 0.8	16.7 ± 1.4	8.1 ± 0.3	8.8 ± 2.1	9.2 ± 0.4
16:1n-7	1.1 ± 0.1	0.8 ± 0.1	4.6 ± 0.6	1.1 ± 0.1	0.9 ± 0.1	1.7 ± 0.2
18:1n-9	5.6 ± 0.5	4.5 ± 0.5	8.2 ± 0.7	3.7 ± 0.1	3.1 ± 0.3	4.4 ± 0.2
18:1n-7	2.3 ± 0.1	2.2 ± 0.2	2.8 ± 0.2	2.7 ± 0.2	2.1 ± 0.2	2.6 ± 0.2
18:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:1n-11	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
20:1n-9	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	2.4 ± 2.2	0.2 ± 0.0
22:1n-11	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.0
22:1n-9	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1n-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
Total polyenoic	54.9 ± 0.8	52.2 ± 2.0	32.8 ± 1.7	60.3 ± 0.5	53.8 ± 2.9	53.7 ± 1.3
n-6 series	21.4 ± 1.4	21.3 ± 1.4	14.9 ± 1.2	20.3 ± 1.0	17.1 ± 1.0	18.6 ± 1.4
18:2n-6	0.5 ± 0.0	0.3 ± 0.0	0.9 ± 0.2	0.8 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
18:3n-6	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0
20:2n-6	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:3n-6	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
20:4n-6[b]	14.2 ± 0.9 A	13.6 ± 1.0 A, B	7.2 ± 0.6 C	13.1 ± 0.6 A, B	12.0 ± 0.8 A, B	11.9 ± 1.2 B
22:4n-6	1.8 ± 0.4	1.2 ± 0.1	1.7 ± 0.2	1.3 ± 0.1	0.9 ± 0.1	1.7 ± 0.2
22:5n-6	4.4 ± 0.4	5.4 ± 0.6	4.3 ± 0.5	4.4 ± 0.4	2.9 ± 0.4	3.6 ± 0.5
n-3 series	33.4 ± 1.4	30.9 ± 1.1	17.9 ± 1.1	40.0 ± 1.1	36.7 ± 2.6	35.1 ± 1.4
18:3n-3	0.4 ± 0.2	0.1 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
18:4n-3	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1
20:4n-3	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.4 ± 0.1
20:5n-3	1.6 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	2.2 ± 0.3	1.4 ± 0.2	2.1 ± 0.4
22:5n-3	4.2 ± 0.3	2.8 ± 0.3	2.6 ± 0.1	6.3 ± 0.6	3.3 ± 0.3	6.0 ± 0.5
22:6n-3	26.8 ± 1.1	26.6 ± 1.1	13.2 ± 1.1	30.5 ± 0.5	31.1 ± 2.2	26.0 ± 1.6

[a]For footnotes, see Table 7
[b]Different letters in the lines of arachidonic acid in Tables 7 and 8 indicate statistical differences ($P < 0.05$).

at comparatively high levels regardless of the condition of the fish, similar to those in muscle PL.

In other viscera, the AA contents in NL from fatty fish (748.5 ± 135.0 mg/100 g) were approximately sixfold higher than those from lean fish (125.5 ± 20.7 mg/100 g). However, NL in fatty fish (32,130 ± 3,948 mg/100 g) was 13-fold higher than in lean fish (2,491 ± 631.7 mg/100g). Consequently, AA levels in NL from lean fish (7.6 ± 1.6%) were significantly higher than those from fatty fish (2.6 ± 0.5%). These results indicate that AA is inferior to other FA, such as MUFA or SFA, as an energy source, and remains unused during the months from winter to summer. Or, AA may be being spared for other roles and functions; the important roles of AA for fish growth have been gradually clarified (48,49). Alternatively, the FA composition in NL of other viscera may be largely influenced by the fish's food sources. In PL, the AA levels fluctuated and were present at higher levels in lean fish (11.9 ± 1.2%) than in fatty fish (7.2 ± 0.6%). This may indicate that the lipids in other viscera, even if they are PL, are influenced by the lipid content of food sources.

Comparative high levels of AA in S. fuscescens *tissues*. In general, n-6 series PUFA, such as AA, are present in undetectable or negligible amounts in marine fish lipids, whereas n-3 series PUFA, such as EPA and DHA, are abundant. These n-3 series PUFA have an important role for membrane fluidity (50), and dominate approximately 50% of TFA in PL (39,51,52). Thus, it is well known that n-3 series PUFA are essential for all marine fish (53–59). These fish may obtain a large part of their n-3 PUFA through the marine food chain, as has been reported for DHA (60–62), because marine fish lack the Δ4- and Δ5-desaturases (53,54). Similar to other marine fishes, *S. fuscescens* contained comparatively high levels of n-3 series PUFA, such as EPA and DHA, in their tissues. These PUFA are probably sourced from the lipids of their diets. *S. fuscescens* tissues also contained high levels of the n-6 series PUFA, AA.

Herbivorous species (including omnivorous species), such

as *Eubalichthys mosaicus*, *Meuschenia freycineti*, *Thamnaconus degeni*, *M. scaber*, and *Pentaceropsis recurvirostris* were also reported to contain high levels of AA (7.4–14.9%) in their muscle TL by Dunstan *et al.* (25). Moreover, they reported that carnivorous *Heterodontus portusjacksoni*, which feed on the macro-algae-consuming sea urchins and sea snails, also contained high levels of AA (13.8%) in their muscle TL (25). A few other reports also suggested that in fishes containing high levels of AA, the AA levels are influenced by dietary lipids (22–25).

Our finding that *S. fuscescens* contained high levels of AA in both organs and stomach contents supports these findings.

From the previous findings and ours, it appears that comparatively high levels of AA observed in those fishes may originate from macro-algae through the marine food chain.

We have only a few reports of the functions of AA in marine fish tissues (48,49). AA may be an essential FA for *S. fuscescens*. They accumulate AA in PL more than in NL, and AA in PL of both muscle and liver was maintained at high levels regardless of the condition of the fish, such as fatty or lean.

S. fuscescens tissues contained high amounts of AA and DHA throughout the year. This suggests that the fish is an available and desirable source for infant food to meet the recently confirmed requirements of both n-3 PUFA and n-6 PUFA for infant growth (8,9,63). Those PUFA were reported to affect neural development and maturation of sensory systems of infants (8,63). Moreover, an increase in the dietary n-3/n-6 FA ratio is desirable to help reduce the risk of cardiovascular disease (64). It is thus important for human health to increase the consumption of n-3 FA. Although there is no officially recommended n-3/n-6 ratio, the suggested ratio is 1:1 from evidence in wild animals and estimated nutrient intake during human evolution (65). The present study shows that the n-3/n-6 ratios of *S. fuscescens* were 1.3–2.5 in muscle, 1.1–2.9 in liver, and 1.0–2.6 in other viscera, indicating that *S. fuscescens* can provide the suggested ratio suitable for the human diet throughout the year.

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cis-4,7,10,*trans*-13-22:4 Fatty Acid Distribution in Phospholipids of Pectinid Species *Aequipecten opercularis* and *Pecten maximus*

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ABSTRACT: The distribution of *cis*-4,7,10,*trans*-13-docosatetraenoic (*c*4,7,10,*t*13-22:4), a peculiar FA previously isolated in the glycerophospholipids of some pectinid bivalves, was investigated in glycerophospholipid classes and subclasses of separated organs (gills, mantle, gonads, and muscle) of the queen scallop *Aequipecten opercularis* and the king scallop *Pecten maximus*. Plasmalogen (Pls) and diacyl + alkyl (Ptd) forms of serine, ethanolamine, and choline glycerophospholipids were isolated by HPLC and their FA compositions analyzed by GC-FID. Pls and Ptd forms of serine glycerophospholipids (PlsSer and PtdSer), and to a lesser extend the Pls form of ethanolamine glycerophospholipids (PlsEtn), were found to be specifically enriched with *c*4,7,10,*t*13-22:4. This specificity was found to decrease in the tested organs in the following order: gills, mantle, gonad, and muscle. In gills, *c*4,7,10,*t*13-22:4 was shown to be the main unsaturated FA of serine glycerophospholipids in both Pls and Ptd forms (23.8 and 19.4 mol%, respectively, for *A. opercularis*, and 21.0 and 26.2 mol% for *P. maximus*). These results represent the first comprehensive report on the FA composition of plasmalogen serine subclass isolated from pectinid bivalves. The specific association of the PlsSer with the *c*4,7,10,*t*13-22:4 for the two pectinid species can be paralleled to the specific association of the PlsSer with the non–methylene interrupted (NMI) FA and 20:1(n-11) observed in mussels, clams, and oysters (Kraffe, E., Soudant, P., and Marty, Y. (2004) Fatty Acids of Serine, Ethanolamine and Choline Plasmalogens in Some Marine Bivalves, *Lipids 39*, 59–66.) This, led us to hypothesize a similar functional significance for *c*4,7,10,*t*13-22:4, NMI FA, and 20:1(n-11) associated with PlsSer subclass of bivalves.

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In *Pecten maximus* larvae and adults (family Pectinidae, class Bivalvia), a new C_{22} PUFA was reported in significant amounts (1–5). This PUFA was later characterized in *P. maximus* and *Argopecten purpuratus* as a new *cis*-4,7,10,*trans*-13-docosatetraenoic FA (*c*4,7,10,*t*13-22:4) (6). This *c*4,7,10,*t*13-22:4, found mainly in glycerophospholipids (Gpl), appeared to be specifically associated with the phosphatidylserine (SerGpl).

The same investigations have also shown that non–methylene-interrupted (NMI) FA were present in only trace amounts in the studied species. Although these NMI FA are seemingly ubiquitous mollusk components (see Ref. 8 for an early review), they were always found in low levels in scallops in comparison with other bivalve mollusks (7–9). Experiments conducted on the mollusks *Scapharca broughtoni* and *Callista brevisiphonata* and the mussel *Mytilus edulis* have shown that marine bivalve mollusks were able to synthesize *de novo* the NMI FA 7,15-22:2 and 7,13-22:2 from carbon-labeled acetate (10,11), whereas it is also established that they have limited or no ability to synthesize PUFA (12–14).

In parallel, it is well established that marine invertebrates are an especially rich source of 1-alkenyl-2-acyl Gpl, commonly called plasmalogens (Pls) (7,15). The presence of a Pls serine subclass (PlsSer), found only in mollusks among all winter-collected invertebrates studied, was first described by Dembitsky and Vaskovsky (16), and confirmed on summer-collected animals by Dembitsky (17). Very little information is available on the FA composition of Pls subclasses from marine bivalves. Only two studies described the FA composition of PlsEtn and PlsCho of *Crassostrea gigas* (18,19), and the PlsSer FA composition was never studied until recently (20). In the present study, the analysis of the FA profiles of both 1-alkenyl-2-acyl (Pls) and 1,2-diacyl + 1-alkyl-2-acyl (Ptd) analogues of Gpl, obtained from the oyster *C. gigas*, the mussel *M. edulis*, and the clam *Ruditapes philippinarum* showed a very consistent high specificity of the aminoplasmalogens PlsSer and PlsEtn, for the NMI and for the 20:1(n-11).

Considering the former results, on the one hand, high levels of NMI were always found in bivalve mollusks except in scallops, and a good specificity of aminoplasmalogens for NMI was shown in mussels, clams, and oysters (which belong to Mytilidae, Ungulinidae, and Ostreidae families, respectively). On the other hand, the peculiar presence of *c*4,7,10,*t*13-22:4 in some scallops, *A. opercularis*, *P. maximus*, and *A. purpuratus* could be associated with very low levels of NMI FA in these species, which belong to the Pectinidae family. The objective of the pre-

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Abbreviations: *c*4,7,10,*t*13-22:4, *cis*-4,7,10,*trans*-13-docosatetraenoic FA; Gpl, glycerophospholipid; LysoPtdCho, natural lysophosphatidylcholine compound (FA in *sn*-1 position); MUFA, monounsaturated FA; NMI, non–methylene interrupted (concerning the present results NMI refers to the sum of *c*5,13-20:2, *c*5,11-20:2, *c*7,15-22:2, *c*7,13-22:2, and *c*7,13,16-22:3); Pls, plasmalogen; PlsCho, 1-alkenyl-2-acyl-glycerophosphatidylcholine; PlsEtn, 1-alkenyl-2-acyl-glycerophosphatidylethanolamine; PlsSer, 1-alkenyl-2-acyl-glycerophosphatidylserine; Ptd, diacyl + alkyl; PtdCho, [1,2-diacyl- + 1-alkyl-2-acyl-] glycerophosphatidylcholine; PtdEtn, [1,2-diacyl- + 1-alkyl-2-acyl-] glycerophosphatidylethanolamine; PtdSer, [1,2-diacyl- + 1-alkyl-2-acyl-] glycerophosphatidylserine; SFA, saturated FA.

sent study was to investigate the repartition of the *c*4,7,10,*t*13-22:4 among the Gpl classes of the different organs of the two scallop species *A. opercularis* and *P. maximus,* (family Pectinidae, class Bivalvia), taking into account the detailed FA composition of the Pls forms of EtnGpl, SerGpl, and ChoGpl.

MATERIALS AND METHODS

Chemicals. Boron trifluoride (BF_3, 10% by wt in methanol) was obtained from Supelco (Saint-Quentin Fallavier, France). Other described reagents and solvents were purchased from Merck (Darmstadt, Germany).

Sample preparation and lipid extraction. Adult *A. opercularis* and *P. maximus* scallops were collected from the Bay of Brest during spring 2001 (between 10 and 20 m depth). The digestive tract was first removed to limit contamination by ingested algae. Then mantle, gills, adductor muscle, and gonads were excised, pooled, weighed, and homogenized with a Dangoumeau homogenizer at –180°C. Three pools of three individuals were analyzed for each of the two species. Lipid extraction was conducted on tissue homogenates with a mixture of $CHCl_3$/MeOH (2:1, vol/vol) following a procedure in the literature (21), modified. To ensure the complete extraction of tissue lipids, a solvent-to-tissue ratio of 70:1 (wt/wt) was used as described by Nelson (22). After removing the organic phase, the residue was re-extracted with a mixture of $CHCl_3$/MeOH (2:1, vol/vol). The final lipid extract was stored at –20°C under nitrogen after 0.01 wt% BHT (antioxidant) was added.

Separation of polar lipids on a silica gel microcolumn. An aliquot of the lipid extract was evaporated to dryness, and lipids were recovered with three washings of 500 µL of $CHCl_3$/MeOH (98:2, vol/vol) and deposited at the top of a silica gel microcolumn (30 × 5 mm i.d., packed with Kieselgel 60 (70-230 mesh, Merck) previously heated at 450°C and deactivated with 6 wt% H_2O (1). Neutral lipids were eluted with 10 mL of $CHCl_3$/MeOH (98:2, vol/vol). The polar lipid fraction was recovered with 20 mL of MeOH and stored at –20°C for later phospholipid class separation by HPLC and FA composition analysis by GC.

Separation of glycerophospholipid classes and FA analysis. Separation of the Gpl classes and subclasses was achieved according to the method previously described (20) using a combination of two successive HPLC separations with two different mobile phases.

(i) Non-acid HPLC separations. A rapid gradient elution separation was achieved on a Merck HPLC system (UV detection at 206 nm) equipped with a Diol phase column (OH-bound silica gel column, Lichrosorb Diol 5 µm, 250 × 4 mm i.d., Merck). An isocratic 9:1 ratio of solvent A (*n*-hexane/2-propanol/water, 40:52:1, by vol) and solvent B (*n*-hexane/2-propanol/water, 40:52:8, by vol) was run for 11 min. This solvent ratio was then followed by a 1-min linear gradient to 100% solvent B. The column was maintained on solvent B for 18 min to complete the separation, then reactivated with 20 min of the initial 9:1 ratio of solvent A to B. The solvent flow rate was 1 mL/min during the entire elution program. This separation allowed the collection of a first fraction (within 3–19 min) containing EtnGpl (1,2-diacyl + 1-alkyl-2-acyl + 1-alkenyl-2-acyl [Pls] Gpl forms) and ChoGpl (1,2-diacyl + 1-alkyl-2-acyl + 1-alkenyl-2-acyl [Pls] Gpl forms), and a second fraction (within 19–25 min) containing cardiolipin, phosphatidylinositol, lysophosphatidylcholine (LysoPtdCho), and SerGpl (1,2-diacyl + 1-alkyl-2-acyl + 1-alkenyl-2-acyl [Pls] Gpl forms).

(ii) Acidic HPLC separations. After evaporation to dryness under nitrogen, first or second fractions were recovered with two washings of 50 µl each of $CHCl_3$/MeOH (98:2, vol/vol) before being manually injected in the 200-µl loop of the second HPLC system. The separation was achieved as previously described (20). This method, based on the use of an acidic mobile phase, allows the hydrolysis of the vinyl ether bond at the *sn*-1 position of the glycerol backbone of the Pls form (4), which creates lyso analogs that elute later than the intact diacyl + alkyl-acyl (Ptd) forms. This offers the possibility of analyzing separately Ptd and Pls subclasses of EtnGpl and ChoGpl from the first fraction, and Ptd and Pls subclasses of SerGpl from the second fraction. Each SerGpl, EtnGpl, and ChoGpl subclass, jointly with cardiolipin, phosphatidylinositol, and LysoPtdCho, was collected and, after transesterification ($MeOH/BF_3$), analyzed by GC for FA composition. FAME obtained were identified and quantified using both polar (CPWAX 52 CB, 50 m × 0.25 mm i.d., 0.2 µm thickness) and nonpolar (CP-Sil 8 CB, 25 m × 0.25 mm i.d., 0.25 µm thickness) capillary columns. FA were expressed as molar percentage of the total FA content of each class or subclass. For PlsSer, PlsEtn, and PlsCho subclasses, the total percentage was adjusted to 50% to take into account the alkenyl chains of the *sn*-1 position hydrolyzed by the acid mobile phase. The quantities of each class and subclass of Gpl were determined from their respective quantitative spectrum of FA obtained by GC. To obtain the molar content of each analyzed fraction, a corrective factor was applied to their respective total FA molar contents: ×1 for the PlsSer, PlsEtn, and PlsCho fractions and for the natural lysoPtdCho fraction; ×1/2 for the PtdEtn, PtdCho, PtdSer, and phosphatidylinositol fractions, and ×1/4 for the cardiolipin fraction. The Ptd fractions were, in that case, assimilated to diacyl fractions.

RESULTS

Gpl composition and Pls content of gills, mantle, gonads and muscle. The detailed Gpl composition of gills, mantle, gonads (male + female), and adductor muscle obtained from *A. opercularis* and *P. maximus* are presented in Table 1. The Gpl subclass compositions of each organ were very similar in *A. opercularis* and *P. maximus.* In gills of the two scallop species the most abundant Gpl subclasses were PlsEtn and PtdCho, respectively 37.6 and 31.7% of the total Gpl in *A. opercularis*, and 34.2 and 33.2% in *P. maximus*. The highest percentage of total plasmalogens (PlsEtn + PlsSer + PlsCho) was found in gills, accounting for 43.3% in *A. opercularis* and 40.4% in *P. maximus*. In mantle, although present at a lower level, Pls totaled respectively 37.4 and 35% of Gpl. In gonads, PlsEtn decreased at the expense of PtdCho, but both remained the most promi-

TABLE 1
Composition of Glycerophospholipids[a] and Plasmalogen Content of EtnGpl[b], SerGpl, and ChoGpl Classes in Separated Organs from *A. opercularis* and *P. maximus*

	Glycerophospholipid Composition (mol %)							
	A. opercularis				*P. maximus*			
	Gills	Mantle	Gonads	Muscle	Gills	Mantle	Gonads	Muscle
PtdEtn[c]	6.1 ± 0.2	6.1 ± 0.1	9.5 ± 0.1	12.6 ± 0.8	5.3 ± 0.9	8.2 ± 0.6	9.5 ± 0.1	17.0 ± 0.3
PlsEtn[d]	37.6 ± 3.7	33.4 ± 2.8	23.4 ± 1.9	18.6 ± 1.3	34.2 ± 0.3	31.4 ± 1.0	25.5 ± 0.2	12.9 ± 1.2
PtdSer	8.6 ± 1.7	14.5 ± 2.2	10.5 ± 3.3	5.1 ± 2.8	9.7 ± 1.4	12.0 ± 2.7	7.9 ± 0.8	8.5 ± 2.4
PlsSer	3.6 ± 2.0	2.5 ± 0.5	2.0 ± 1.0	0.7 ± 0.2	4.5 ± 1.5	1.9 ± 0.7	1.2 ± 0.4	0.6 ± 0.2
PtdCho	31.7 ± 0.1	32.5 ± 0.6	41.8 ± 4.4	53.4 ± 2.9	33.2 ± 5.1	36.3 ± 0.1	45.1 ± 2.2	49.4 ± 3.0
PlsCho	2.1 ± 0.2	1.5 ± 0.1	1.4 ± 1.2	0.5 ± 0.1	1.7 ± 0.8	1.7 ± 0.7	0.9 ± 0.6	0.9 ± 0.3
Phosphatidylinositol	5.0 ± 0.5	5.0 ± 1.2	6.6 ± 1.2	6.4 ± 0.7	7.2 ± 2.3	5.8 ± 0.7	5.5 ± 0.2	8.7 ± 2.8
Cardiolipin	0.7 ± 0.3	0.6 ± 0.2	1.8 ± 0.7	0.6 ± 0.1	0.9 ± 0.5	0.9 ± 0.2	2.2 ± 1.0	0.7 ± 0.4
LysoPtdCho	4.5 ± 1.0	3.9 ± 1.9	3.0 ± 1.5	2.1 ± 1.3	3.2 ± 0.8	1.7 ± 0.5	2.2 ± 0.7	1.3 ± 0.2
Total Plasmalogen	43.3 ± 3.5	37.4 ± 3.2	26.8 ± 0.3	19.8 ± 1.4	40.4 ± 1.9	35.0 ± 1.1	27.6 ± 2.6	14.4 ± 3.4
Plasmalogen Content of the Class[e]								
EtnGpl	86.0 ± 0.7	84.6 ± 1.2	71.1 ± 1.7	59.6 ± 0.1	86.6 ± 1.8	79.3 ± 0.7	72.9 ± 0.2	43.1 ± 2.7
SerGpl	29.5 ± 7.4	14.7 ± 0.8	16.0 ± 3.7	12.1 ± 1.9	31.7 ± 4.2	13.7 ± 2.3	13.2 ± 2.5	6.6 ± 0.1
ChoGpl	6.2 ± 0.7	4.4 ± 0.4	3.2 ± 2.7	0.9 ± 0.2	4.9 ± 3.0	4.5 ± 0.7	2.0 ± 1.4	1.8 ± 0.9

[a]Expressed as mol % of total moles of glycerophospholipids, calculated as indicated in text. Results are mean ± SD (n = 3).
[b]EtnGpl (or SerGpl or ChoGpl) refers to the pool of the [diacyl + alkyl acyl + alkenyl acyl (plasmalogen)] forms of glycerophosphatidylethanolamine (or serine or choline) class.
[c]« Ptd » refers to diacyl form + alkyl acyl form (see text).
[d]« Pls » refers to alkenyl acyl (plasmalogen) form.
[e]Proportion of the plasmalogen form relatively to the whole of a same class forms.

nent Gpl components, respectively 23.4 and 41.8% in *A. opercularis*, and 25.5 and 45.1% in *P. maximus*. In the adductor muscle this tendency was accentuated and PtdCho amounted to approximately half of the Gpl content, while total Pls accounted only for 19.8% in *A. opercularis* and 14.4% in *P. maximus*. In parallel, and for both species, highest Pls proportions in EtnGpl, SerGpl, and ChoGpl were found in gills, accounting for more than 86.0, 29.5, and 4.9%, respectively. These values decreased in mantle and gonads, and the lowest percentages were observed in the muscle, for both species.

FA composition of the different forms of EtnGpl, SerGpl, and ChoGpl of gills, mantle, gonads, and muscle. In separated organs of the two pectinid species, five major FA (16:0, 18:0, 20:4(n-6), 20:5(n-3), 22:6(n-3), and *c*4,7,10,*t*13-22:4) represented 70–90% of the total FA in the Pls (*sn*-2 position) and Ptd forms (*sn*-1 and *sn*-2 positions) of ethanolamine, serine, and choline Gpl (Tables 2 and 3). Total saturated FA (SFA) content was found to be very low in PlsEtn and slightly higher in PlsSer (respectively <2.8 and <18% in *A. opercularis*, and <2.1 and <15.0% in *P. maximus*), whereas it was higher in PlsCho for all organs <30.4% in *A. opercularis* and <22.1% in *P. maximus*. In Ptd fractions of EtnGpl, SerGpl, and ChoGpl, SFA content ranged from 28.4 to 46.7%. SFA were dominated by the 16:0 and 18:0 in all fractions. Total monounsaturated FA (MUFA) were low in Pls fractions of all organs, <5.8% in *A. opercularis* and <5.1% in *P. maximus*, and in Ptd fractions they were <12.1% in *A. opercularis* and <14.9% in *P. maximus*. Long-chain PUFA with 20 and 22 carbons were found to be the dominant PUFA in the Pls and Ptd fractions. They were mainly constituted by 20:4(n-6), 20:5(n-3), 22:6(n-3), and *c*4,7,10,*t*13-22:4. Only very low levels of NMI FA were detected among the different organs of the two species (Tables 2 and 3).

c4,7,10,t13-22:4 distribution in Gpl and in the different forms of EtnGpl, SerGpl, and ChoGpl. The content of *c*4,7,10,*t*13-22:4 in Gpl was found to range from the highest value in gills (9.9 and 9.3% in *A. opercularis* and *P. maximus*, respectively) to the lowest value in muscle (1.2 and 1.3% in *A. opercularis* and *P. maximus*, respectively) (Table 4). For all organs tested, the *c*4,7,10,*t*13-22:4 FA was found to be preferentially located in SerGpl, EtnGpl, and ChoGpl, whereas it was detected in only trace amounts in the other analyzed Gpl classes, phosphatidylinositol, cardiolipin, and lysoPtdCho (data not shown). Variations of *c*4,7,10,*t*13-22:4 proportions in the Pls and Ptd forms of SerGpl, EtnGpl, and ChoGpl among the different organs of *A. opercularis* and *P. maximus* are summarized in Figure 1. In all organs of both species the highest proportions of *c*4,7,10,*t*13-22:4 were found in PtdSer and PlsSer fractions. When comparing organs, gills showed the highest percentages of *c*4,7,10,*t*13-22:4, similarly in Pls and Ptd forms, reaching 19.4 and 23.8% of total FA, respectively, for PtdSer and PlsSer fractions in *A. opercularis,* and 26.2 and 21.0% in *P. maximus*. In the mantle, equivalent levels of *c*4,7,10,*t*13-22:4

TABLE 2
FA Composition of EtnGpl[a], SerGpl, and ChoGpl Subclasses in Separated Organs from *A. opercularis*[b]

	Gills						Mantle					
	EtnGpl		SerGpl		ChoGpl		EtnGpl		SerGpl		ChoGpl	
FA	Ptd[c]	Pls[d]	Ptd	Pls	Ptd	Pls	Ptd	Pls	Ptd	Pls	Ptd	Pls
16:0	13.2 ± 1.8	0.5 ± 0.2	5.0 ± 2.3	2.8 ± 0.9	20.2 ± 0.2	6.4 ± 1.2	12.3 ± 0.7	0.6 ± 0.2	3.5 ± 0.8	3.2 ± 0.7	23.3 ± 0.9	7.8 ± 1.4
18:0	17.5 ± 0.2	0.5 ± 0.2	35.8 ± 0.7	5.7 ± 1.1	3.0 ± 0.2	6.4 ± 3.9	16.2 ± 0.3	0.6 ± 0.3	40.0 ± 2.1	9.5 ± 3.1	2.2 ± 0.2	9.3 ± 4.7
20:1(n-11)	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.2	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
20:4(n-6)	10.3 ± 0.6	8.9 ± 0.9	7.0 ± 0.5	3.2 ± 1.0	3.6 ± 0.1	2.9 ± 0.5	11.1 ± 0.8	7.8 ± 0.6	5.2 ± 1.1	5.0 ± 1.2	3.7 ± 0.2	2.4 ± 0.4
20:5(n-3)	20.9 ± 1.3	7.7 ± 0.1	8.3 ± 0.3	3.4 ± 0.4	13.8 ± 0.6	4.7 ± 0.9	15.6 ± 2.4	4.6 ± 0.5	7.1 ± 1.4	3.4 ± 0.4	11.4 ± 1.8	3.0 ± 1.2
NMI[e]	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
4,7,10,*t*13-22:4	3.8 ± 0.1	10.3 ± 0.8	19.4 ± 1.7	23.8 ± 0.6	2.6 ± 0.2	2.8 ± 0.6	1.5 ± 0.1	2.4 ± 0.1	8.8 ± 1.6	7.7 ± 1.7	0.6 ± 0.1	0.6 ± 0.5
22:6(n-3)	19.2 ± 0.3	16.4 ± 0.3	13.8 ± 0.5	5.3 ± 1.4	24.9 ± 0.6	16.0 ± 1.4	26.7 ± 0.8	30.5 ± 0.8	20.7 ± 2.5	15.1 ± 1.8	24.1 ± 0.7	12.4 ± 4.5
Total SFA	35.9 ± 2.0	1.2 ± 0.4	44.5 ± 3.1	9.5 ± 2.6	32.5 ± 0.4	14.8 ± 4.1	35.8 ± 3.3	1.4 ± 0.4	46.5 ± 4.0	14.3 ± 4.0	39.3 ± 1.5	22.5 ± 6.0
Total MUFA	6.1 ± 1.2	1.7 ± 0.2	3.9 ± 0.3	2.2 ± 0.1	10.3 ± 0.3	3.4 ± 0.6	6.9 ± 1.3	1.0 ± 0.1	4.8 ± 1.5	3.0 ± 1.0	12.1 ± 0.7	5.8 ± 1.9
Total PUFA	57.9 ± 0.8	47.1 ± 0.6	51.6 ± 2.9	38.3 ± 2.6	57.2 ± 0.5	31.8 ± 4.5	57.3 ± 4.6	47.6 ± 0.6	48.7 ± 5.7	32.7 ± 5.0	48.6 ± 1.0	21.7 ± 6.1

	Gonads						Muscle					
	EtnGpl		SerGpl		ChoGpl		EtnGpl		SerGpl		ChoGpl	
FA	Ptd[c]	Pls[d]	Ptd	Pls	Ptd	Pls	Ptd	Pls	Ptd	Pls	Ptd	Pls
16:0	16.6 ± 0.7	1.3 ± 0.6	11.3 ± 2.0	6.5 ± 2.5	25.6 ± 0.9	16.6 ± 0.1	15.0 ± 0.7	0.6 ± 0.1	4.5 ± 0.9	7.7 ± 0.8	20.7 ± 0.4	9.6 ± 0.1
18:0	12.7 ± 1.1	1.3 ± 0.3	30.2 ± 2.9	8.5 ± 0.4	3.5 ± 0.3	10.4 ± 0.1	8.2 ± 0.5	0.4 ± 0.1	26.6 ± 0.9	9.0 ± 2.4	1.0 ± 0.1	5.3 ± 1.6
20:1(n-11)	0.3 ± 0.0	0.1 ± 0.0	0.8 ± 0.7	0.5 ± 0.4	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.4 ± 0.0	0.5 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
20:4(n-6)	4.8 ± 0.2	5.7 ± 0.3	4.5 ± 1.5	3.4 ± 0.7	2.5 ± 0.3	1.5 ± 0.1	5.7 ± 0.7	4.5 ± 0.2	4.0 ± 0.6	4.0 ± 1.3	2.7 ± 0.3	2.4 ± 0.5
20:5(n-3)	31.9 ± 0.8	22.1 ± 1.0	16.0 ± 2.6	9.8 ± 1.8	20.8 ± 1.9	6.5 ± 0.7	28.5 ± 0.2	11.2 ± 1.6	10.5 ± 0.6	6.9 ± 1.3	21.5 ± 0.1	8.9 ± 2.9
NMI[e]	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.4	0.4 ± 0.6	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	1.0 ± 1.4	0.1 ± 0.0	0.0 ± 0.0
4,7,10,t13-22:4	1.3 ± 0.1	1.7 ± 0.2	9.5 ± 2.1	3.0 ± 0.7	0.9 ± 0.1	0.6 ± 0.2	0.8 ± 0.0	1.1 ± 0.1	8.6 ± 0.8	2.9 ± 1.2	0.2 ± 0.0	0.5 ± 0.7
22:6(n-3)	11.5 ± 0.3	13.9 ± 1.9	12.9 ± 2.1	9.5 ± 3.9	17.6 ± 0.6	5.6 ± 0.6	25.0 ± 2.1	29.4 ± 1.8	33.3 ± 1.1	14.4 ± 0.8	16.9 ± 0.5	14.8 ± 3.1
Total SFA	33.5 ± 1.2	2.8 ± 1.1	45.7 ± 2.0	16.1 ± 3.1	35.8 ± 0.4	30.4 ± 2.9	29.0 ± 1.7	1.1 ± 0.3	34.3 ± 1.3	18.0 ± 1.5	35.4 ± 0.4	19.7 ± 0.5
Total MUFA	10.5 ± 1.0	1.5 ± 0.6	5.9 ± 1.2	4.8 ± 1.5	8.9 ± 0.4	2.9 ± 0.0	6.4 ± 0.3	0.8 ± 0.1	3.9 ± 0.5	1.1 ± 1.5	11.9 ± 0.4	2.2 ± 3.1
Total PUFA	56.0 ± 0.2	45.6± 1.8	48.3 ± 2.7	29.1 ± 1.6	55.4 ± 0.7	16.7 ± 2.9	64.6 ± 1.9	48.1 ± 0.5	61.8 ± 0.9	31.0 ± 0.0	52.6 ± 0.1	28.2 ± 3.5

[a]For abbreviations see Table 1.
[b]Results expressed as mol % are mean ± SD (n = 3).
[c]« Ptd » refers to both *sn*-1 and *sn*-2 fatty acyl chains of the diacyl form + *sn*-2 fatty acyl chains of the alkyl form.
[d]« Pls » refers to *sn*-2 fatty acyl chains of the plasmalogen form, and total percent was adjusted to 50% to take into account the alkenyl chains of the *sn*-1 position hydrolyzed by the acid mobile phase as described in text.
[e]« NMI » refers to sum of all non-methylene-interrupted FA: 5,13-20:2; 5,11-20:2; 7,15-22:2; 7,13-22:2; 7,13,16-22:3.

were also found between PtdSer and PlsSer, but these proportions were lower than in gills (about 9 and 13%, respectively, in *A. opercularis* and *P. maximus*). Similar levels of *c*4,7,10,*t*13-22:4 were also found in gonad and muscle, but in PtdSer only (from 7.1 to 9.5% in PtdSer of both species). Lower percentages of *c*4,7,10,*t*13-22:4 were found in PlsSer of these organs (between 2.6 and 4.8% in both species). In addition, noticeable levels of *c*4,7,10,*t*13-22:4 were found in gill PlsEtn (10.3 and 7.7% in *A. opercularis* and *P. maximus*, respectively).

DISCUSSION

Examination of the *c*4,7,10,*t*13-22:4 repartition within the Pls and Ptd forms of SerGpl, EtnGpl, and ChoGpl let us to report on the specific distribution of this FA in these Gpl subclasses. The *c*4,7,10,*t*13-22:4 has been shown to be especially concentrated in PlsSer and PtdSer, and to a lesser extent in PlsEtn. This specific association was evidenced in all tissues analyzed, but proportions of the *c*4,7,10,*t*13-22:4 in these subclasses were the highest in gills and the lowest in the adductor muscle. In gills, the *c*4,7,10,*t*13-22:4 was shown to be the main unsaturated FA of the SerGpl class, both in Pls and Ptd forms. To date, very little information was available on the FA composition of Pls subclasses from marine bivalves, and particularly on the FA composition of the PlsSer detected only in mollusks (18). Indeed, this is the first study reporting on the FA composition of PlsSer subclass in pectinid bivalves.

Using the same methodology, a previous analysis of the oyster *C. gigas*, the mussel *M. edulis*, and the clam *R. philip-*

TABLE 3
FA Composition of EtnGpl[a], SerGpl, and ChoGpl Subclasses in Separated Organs from *P. maximus*[b]

	Gills						Mantle					
	EtnGpl		SerGpl		ChoGpl		EtnGpl		SerGpl		ChoGpl	
FA	Ptd[c]	Pls[d]	Ptd	Pls	Ptd	Pls	Ptd	Pls	Ptd	Pls	Ptd	Pls
16:0	12.3 ± 0.3	0.4 ± 0.1	3.2 ± 0.2	3.8 ± 0.5	18.1 ± 1.9	4.9 ± 0.9	16.1 ± 0.4	0.5 ± 0.2	4.0 ± 0.8	4.2 ± 1.2	22.9 ± 0.7	6.3 ± 0.7
18:0	19.6 ± 2.3	0.4 ± 0.0	34.9 ± 0.8	3.8 ± 0.6	3.5 ± 0.4	4.3 ± 1.5	16.7 ± 0.7	0.4 ± 0.1	36.6 ± 0.3	4.1 ± 1.4	2.4 ± 0.3	5.2 ± 0.7
20:1(n-11)	0.3 ± 0.1	0.8 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.8 ± 0.1	1.6 ± 0.9	0.1 ± 0.0	0.0 ± 0.0
20:4(n-6)	9.2 ± 0.6	6.9 ± 0.1	6.8 ± 0.5	3.1 ± 0.2	4.3 ± 0.1	3.9 ± 0.1	12.0 ± 0.4	8.2 ± 0.6	8.1 ± 1.1	4.4 ± 0.8	4.1 ± 0.1	3.4 ± 0.5
20:5(n-3)	19.4 ± 0.6	5.1 ± 0.3	7.4 ± 0.2	2.3 ± 0.3	13.6 ± 0.8	6.4 ± 0.7	19.3 ± 0.9	5.3 ± 0.1	7.3 ± 0.6	4.0 ± 0.2	11.9 ± 0.5	5.1 ± 0.5
NMI[e]	0.9 ± 0.2	3.2 ± 0.0	1.7 ± 0.2	2.0 ± 0.1	0.4 ± 0.1	0.3 ± 0.3	0.2 ± 0.1	1.3 ± 0.1	0.8 ± 0.1	2.3 ± 1.7	0.1 ± 0.1	0.2 ± 0.2
4,7,10,t13-22:4	3.4 ± 0.5	7.7 ± 0.6	26.2 ± 1.8	21.0 ± 2.7	2.7 ± 0.2	1.9 ± 0.3	1.5 ± 0.1	3.2 ± 1.7	12.9 ± 2.4	13.0 ± 4.8	1.4 ± 0.2	2.4 ± 0.4
22:6(n-3)	13.0 ± 0.8	18.2 ± 0.3	7.8 ± 0.5	5.9 ± 0.4	27.0 ± 1.4	18.3 ± 1.1	20.0 ± 0.5	27.1 ± 0.8	17.9 ± 1.3	9.3 ± 0.2	24.9 ± 0.2	16.1 ± 1.1
Total SFA	36.3 ± 0.3	0.9 ± 0.1	40.8 ± 0.3	9.0 ± 2.0	28.4 ± 2.4	12.0 ± 2.6	38.2 ± 0.9	1.0 ± 0.5	44.1 ± 1.2	9.8 ± 2.8	34.9 ± 0.8	15.8 ± 1.4
Total MUFA	14.9 ± 0.1	3.1 ± 0.1	4.9 ± 0.4	3.4 ± 0.5	11.6 ± 0.6	2.9 ± 1.0	6.0 ± 0.5	1.7 ± 0.2	5.8 ± 4.3	5.1 ± 2.3	13.1 ± 0.4	2.7 ± 1.1
Total PUFA	48.8 ± 0.3	45.9 ± 0.2	54.3 ± 0.6	37.7 ± 2.5	60.0 ± 2.3	35.1 ± 3.5	55.7 ± 0.7	47.3 ± 0.5	50.1 ± 4.4	35.1 ± 5.1	52.0 ± 1.0	31.6 ± 0.3

	Gonads						Muscle					
	EtnGpl		SerGpl		ChoGpl		EtnGpl		SerGpl		ChoGpl	
FA	Ptd[c]	Pls[d]	Ptd	Pls	Ptd	Pls	Ptd	Pls	Ptd	Pls	Ptd	Pls
16:0	17.1 ± 1.1	0.8 ± 0.2	11.2 ± 1.5	5.3 ± 2.1	24.3 ± 0.6	11.1 ± 4.9	20.6 ± 1.1	1.2 ± 0.4	5.8 ± 0.2	6.6 ± 0.2	26.7 ± 0.1	12.9 ± 3.7
18:0	13.8 ± 1.9	0.7 ± 0.4	32.1 ± 2.2	7.1 ± 2.7	3.3 ± 0.3	6.6 ± 3.3	7.5 ± 0.4	0.7 ± 0.1	25.3 ± 0.9	6.4 ± 0.2	1.0 ± 0.0	8.0 ± 4.4
20:1(n-11)	0.3 ± 0.0	0.2 ± 0.1	0.7 ± 0.2	0.7 ± 0.4	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	1.0 ± 0.1	0.5 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
20:4(n-6)	5.2 ± 0.4	5.3 ± 0.4	3.8 ± 0.6	3.9 ± 0.1	2.6 ± 0.3	1.8 ± 0.3	3.9 ± 0.2	2.4 ± 0.2	2.9 ± 0.3	2.5 ± 0.2	2.0 ± 0.2	0.4 ± 0.8
20:5(n-3)	31.6 ± 1.1	20.2 ± 2.6	17.6 ± 0.7	11.4 ± 0.2	19.8 ± 1.6	7.8 ± 1.8	28.8 ± 0.8	6.0 ± 0.5	8.1 ± 0.8	5.4 ± 0.1	20.8 ± 0.2	7.0 ± 0.7
NMI[e]	0.1 ± 0.0	1.2 ± 0.9	0.5 ± 0.3	1.4 ± 2.0	0.2 ± 0.1	0.4 ± 0.7	0.1 ± 0.1	0.6 ± 0.3	0.4 ± 0.3	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
4,7,10,t13-22:4	0.9 ± 0.5	1.2 ± 0.4	7.1 ± 1.6	2.6 ± 1.4	0.7 ± 0.2	0.5 ± 0.2	1.0 ± 0.1	1.4 ± 0.3	9.0 ± 0.3	4.2 ± 0.2	0.1 ± 0.0	0.2 ± 0.4
22:6(n-3)	11.2 ± 0.1	16.7 ± 3.1	14.5 ± 1.1	9.7 ± 2.7	20.1 ± 2.6	10.3 ± 4.4	23.8 ± 1.5	33.1 ± 1.8	35.8 ± 1.1	17.0 ± 0.3	21.1 ± 0.1	16.7 ± 5.0
Total SFA	33.7 ± 1.4	1.7 ± 0.7	46.7 ± 0.7	14.4 ± 5.6	33.4 ± 1.7	21.0 ± 8.1	33.2 ± 1.3	2.1 ± 0.4	34.9 ± 0.8	15.0 ± 0.1	35.4 ± 0.1	22.1 ± 6.7
Total MUFA	10.9 ± 0.5	1.8 ± 0.3	4.9 ± 0.5	4.3 ± 0.8	9.3 ± 0.9	3.6 ± 1.1	5.7 ± 0.4	2.6 ± 0.8	4.9 ± 0.4	4.2 ± 0.1	9.9 ± 0.4	1.2 ± 2.1
Total PUFA	55.5 ± 1.0	46.6 ± 0.7	48.5 ± 0.7	31.3 ± 4.7	57.3 ± 1.4	25.5 ± 9.4	61.0 ± 1.8	45.3 ± 1.1	60.2 ± 0.7	30.7 ± 0.1	54.7 ± 0.2	26.7 ± 6.3

[a–e]For footnotes see Table 2.

pinarum indicated that Pls forms of EtnGpl and SerGpl were found to be specifically enriched with NMI FA and 20:1(n-11) (20). Interestingly, this trend was particularly obvious in PlsSer, in which NMI FA and 20:1(n-11) were found to be, respectively, the predominant PUFA and monounsaturated FA for the three species. Moreover, this specificity was observed in all the analyzed organs of the clams, with the highest levels measured in gills, and the lowest measured in the muscle (20). Consequently one can draw a parallel between the specific FA pattern of PlsSer subclass enriched with NMI FA and 20:1(n-11) in gills of the clams, and with that of the pectinid species *A. opercularis* and *P. maximus* enriched with *c*4,7,10,*t*13-22:4 (present study). Several investigations showed that bivalve species, including mussels, clams, and oysters, generally contain significant amounts of 22:2 NMI FA (7,15-22:2 and 7,13-22:2), and to a lesser extent 20:2 NMI (5,13-20:2 and 5,11-20:2) (23–26). On the other hand, reports on scallop FA compositions (8,9) indicated that the levels of NMI FA were very low in pectinids in comparison with other members of the class Bivalvia. Moreover, *c*4,7,10,*t*13-22:4 found in scallops *A. purpuratus* and *P. maximus* was not detected in the non-pectinid bivalve species such as *Glycymeris glycymeris*, *M. edulis*, *Ostrea edulis*, *C. gigas*, *Tapes decussatus*, and *Crassostrea edule* (6, 20). This suggests some functional and/or structural analogies

TABLE 4
4,7,10,*t*13-22:4 Content in Glycerophospholipids of Separated Organs of *A. opercularis* and *P. maximus* (in mol % of the Total FA of Glycerophospholipid)

	Gills	Mantle	Gonads	Muscle
A. opercularis	9.9 ± 1.4	3.5 ± 0.2	2.0 ± 0.1	1.2 ± 0.1
P. maximus	9.3 ± 0.1	4.7 ± 1.0	1.7 ± 0.5	1.3 ± 0.3

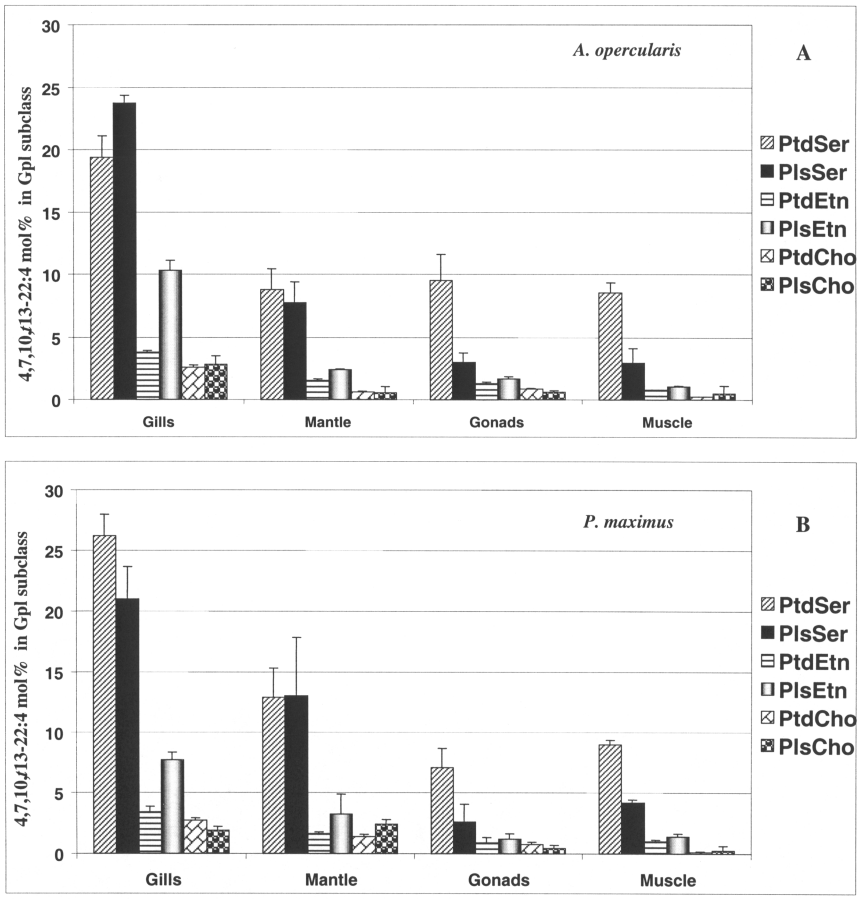

FIG. 1. Percent composition of *c*4,7,10,*t*13-22:4 (in mol%, mean ± SD, $n = 3$) from serine (Ser), ethanolamine (Etn), and choline (Cho) glycerophospholipid subclasses of (A) *A. opercularis* and (B) *P. maximus* organs.

between NMI FA in non-pectinid bivalves and *c*4,7,10, *t*13-22:4 in pectinid bivalves.

In addition, we are also proposing that these peculiar FA (NMI, 20:1(n-11), and *c*4,7,10,*t*13-22:4) may be of endogenous origin even though biosynthesis of long-chain PUFA is considered to be nonexistent or limited in mollusks (12,14). Indeed, as first proposed by Ackman and Hooper (23), and demonstrated by Zhukova and Svetashev (27), and Zhukova (11), on *S. broughtoni* and *M. edulis*, bivalve mollusks are able to synthesize NMI FA. The possibility that 20:1(n-11) and *c*4,7,10,*t*13-22:4 could also be of endogenous origin must be considered, as previously evoked and discussed (6,20). Unfortunately, there is not yet demonstration of that in literature.

In both pectinid bivalves analyzed in the present study, the highest level of *c*4,7,10,*t*13-22:4 was found in gills. Previous investigations on NMI FA distribution in various tissues of bivalve mollusks have indicated the same uneven distribution with highest levels found in gills (7,20,26). Thus, the requisite presence of a high level of *c*4,7,10,*t*13-22:4 or NMI, and 20:1(n-11), in bivalve gills in comparison with muscle, must be further questioned and investigated in terms of functional and structural roles. Nevertheless, some differences between pectinid and non-pectinid species can also be noticed. In *A. opercularis* and *P. maximus* *c*4,7,10,*t*13-22:4 was found to be highly associated with PtdSer. In comparison, in the non-pectinid species analyzed, NMI FA and 20:1(n-11) were mainly associated with PlsSer, and PlsEtn. In pectinids, the greater association of *c*4,7,10,*t*13-22:4 with the serine polar head, in comparison with non-pectinids, is still unclear. However, because the specific association of the peculiar FA with an aminoglycerophospholipid is conserved for all studied bivalve species, one may conclude that *c*4,7,10,*t*13-22:4, NMI FA, and 20:1(n-11) are asymmetrically distributed in the lipid bilayer, and are involved in specific cellular function of the inner membrane. Although these peculiar FA are structurally different, they could play a similar role in membrane functions of different bivalve species.

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METHOD

Synthesis of 3-Oxalinolenic Acid and β-Oxidation-Resistant 3-Oxa-oxylipins

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ABSTRACT: 3-Oxalinolenic acid (3-oxa-9(*Z*),12(*Z*),15(*Z*)-octadecatrienoic acid or (6(*Z*),9(*Z*),12(*Z*)-pentadecatrienyloxy)acetic acid) was synthesized from 5(*Z*),8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosapentaenoic acid by a sequence involving the C15 aldehyde 3(*Z*),6(*Z*),9(*Z*),12(*Z*)-pentadecatetraenal as a key intermediate. Conversion of the aldehyde by isomerization and two steps of reduction afforded 6(*Z*),9(*Z*),12(*Z*)-pentadecatrienol, which was coupled to bromoacetate to afford after purification by HPLC >99%-pure 3-oxalinolenic acid in 10–15% overall yield. 3-Oxalinolenic acid was efficiently oxygenated by soybean lipoxygenase-1 into 3-oxa-13(S)-hydroperoxy-9(*Z*),11(E),15(*Z*)-octadecatrienoic acid, and this hydroperoxide could be further converted chemically into 3-oxa-13(S)-hydroxy-9(*Z*),11(E),15(*Z*)-octadecatrienoic acid and 3-oxa-13-oxo-9(*Z*),11(E),15(*Z*)-octadecatrienoic acid. The 3-oxa-hydroperoxide also served as the substrate for the plant enzymes allene oxide synthase, divinyl ether synthase, and hydroperoxide lyase to produce 3-oxa-12-oxo-10,15(*Z*)-phytodienoic acid and other 3-oxa-oxylipins that were characterized by MS. 3-Oxalinolenic acid was not oxygenated by 9-lipoxygenase from tomato but was converted at a slow rate into 3-oxa-9(S)-hydroperoxy-10(E),12(*Z*),15(*Z*)-octadecatrienoic acid by recombinant maize 9-lipoxygenase. Recombinant α-dioxygenase-1 from *Arabidopsis thaliana* catalyzed the conversion of 3-oxalinolenic acid into a 2-hydroperoxide, which underwent spontaneous degradation into a mixture of 6,9,12-pentadecatrienol and 6,9,12-pentadecatrienyl formate. A novel α-dioxygenase from the moss *Physcomitrella patens* was cloned and expressed and was found to display the same activity with 3-oxalinolenic acid as *Arabidopsis thaliana* α-dioxygenase-1. Lipoxygenase-generated 3-oxa-oxylipins are resistant toward β-oxidation and have the potential for displaying enhanced biological activity in situations where activity is limited by metabolic degradation.

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FA hydroperoxides can be enzymatically produced in plants by lipoxygenase- or α-dioxygenase-catalyzed oxygenations (1,2). Further conversion by secondary enzymes such as allene oxide synthase, allene oxide cyclase, divinyl ether synthase, and hydroperoxide lyase results in an array of oxygenated derivatives collectively called oxylipins. Interest in oxylipins stems from their involvement in plant development and their role as defensive agents in plants attacked by fungi, insects, and bacteria (3,4). Assays of the growth-inhibitory actions of oxylipins on fungi and bacteria have shown impressive effects in many studies (5–7); however, interpretation of the results is often complicated by the fact that active oxylipins have limited stability (6,7). Degradation of certain oxylipins, such as hydroperoxides, can be attributed to inherent chemical instability, whereas others, such as ketodienes and ketotrienes, react chemically with glutathione and other biomolecules, and in this way escape from the test system. Furthermore, a large group of oxylipins are actively metabolized by the microorganism under study. In recent work on the *in vitro* antimicrobial effects of oxylipins, the β-oxidation system for degradation of the carbon chain of FA and FA derivatives was identified as an important pathway for inactivation of exogenously added oxylipins (7). Substitution of the β-methylene group of FA with oxygen (forming 3-oxa FA) affords derivatives that cannot be degraded by mitochondrial or peroxisomal β-oxidation (8,9). The present work describing synthesis of 3-oxalinolenic acid and 3-oxa-oxylipins was carried out with the goal of generating oxylipin analogs resistant to β-oxidation and therefore potentially displaying enhanced biological activity.

EXPERIMENTAL PROCEDURES

FA and reference oxylipins. 5(*Z*),8(*Z*),11(*Z*),14(*Z*),17(*Z*)-Eicosapentaenoic acid was purchased from Nu-Chek-Prep (Elysian, MN). The oxylipins used in the present work, including linoleic and linolenic acid hydroperoxides, divinyl ether FA, and 12-oxo-10,15(*Z*)-phytodienoic acid (12-oxo-PDA), were purchased from Larodan Fine Chemicals (Malmö, Sweden).

*Methyl 5,6-dihydroxy-8(Z),11(Z),14(Z),17(Z)-eicosatetraenoate (**2**).* The procedures described previously by Skattebol and others (10,11) were used with slight modifications. In a typical run, 5(*Z*),8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosapentaenoic acid (**1**; 1.6 g; 5.3 mmol) in THF (80 mL) was treated

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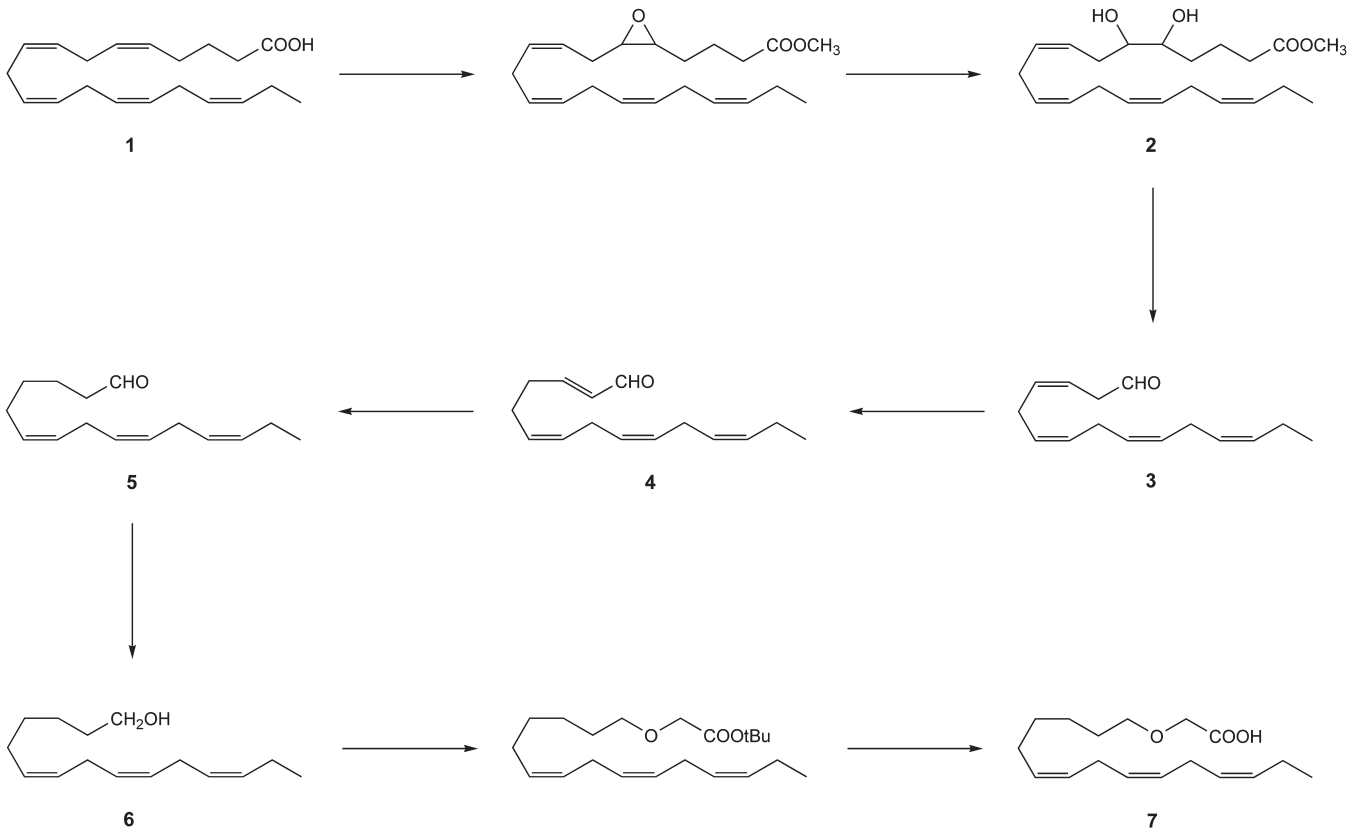
Abbreviations: At-DOX1, recombinant α-dioxygenase-1 from *Arabidopsis thaliana*; 12-oxo-PDA, 12-oxo-10,15(*Z*)-phytodienoic acid; Pp-DOX, recombinant α-dioxygenase from *Physcomitrella patens*.

at 0°C with $KHCO_3$ (3.8 g in 132 mL of water) and sodium iodide and iodine (5.6 g and 9.4 g, respectively, in 80 mL of THF). After adding a few crystals of BHT and purging with argon, the solution was stirred at 23°C for 22 h. The iodolactone obtained by extraction with diethyl ether was dissolved in THF (160 mL) and treated with LiOH (1.4 g) in 108 mL of water. After stirring at 23°C for 1 h, crude *cis*-5,6-epoxy-8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosatetraenoic acid was isolated by extraction with diethyl ether, and its methyl ester was prepared by treatment with diazomethane. Conversion of the epoxyester into the corresponding 5,6-diol (Scheme 1) was effected by treatment with formic acid/acetic anhydride followed by K_2CO_3 in methanol as described (11). Purification by open column silicic acid chromatography (elution with diethyl ether/hexane [8:2, vol/vol]) afforded methyl 5,6-dihydroxy-8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosatetraenoate as a colorless oil (1.2 g; yield, 64%). Analysis of the Me_3Si derivative by GC-MS showed a single eluted peak. MS showed fragments at *m/z* 494 (6%, M^+), 463 (4; $M^+ - 31$; loss of OMe), 305 (23; Me_3SiO^+=CH-CH($OSiMe_3$)-$(CH_2)_3$-COOMe), 215 (37; 305-90), 203 (76; Me_3SiO^+=CH-$(CH_2)_3$-COOMe), and 73 (100; Me_3Si^+).

*2(E),6(Z),9(Z),12(Z)-Pentadecatetraenal (**4**).* The previous product **2** (1.2 g; 3.4 mmol) in acetone (120 mL), water (30 mL), and glacial acetic acid (9 mL) was treated with sodium periodate (3.6 g) at 23°C under magnetic stirring for 1 h. Extraction with hexane afforded 3(*Z*),6(*Z*),9(*Z*),12(*Z*)-pentadecatetraenal (**3**) as an oil producing a single peak upon GC-MS analysis. MS analysis gave *m/z* 218 (<1%; M^+), 189 (1; $M^+ - 29$; loss of C_2H_5), 135 (6), 122 (16), 105 (24), 93 (49), 91 (55), and 79 (100). The aldehyde was dissolved in THF (60 mL) and isomerized by treatment with 70% perchloric acid (2 mL) at 23°C for 1 h. Diethyl ether was added, and the solution was washed with water and dried over $MgSO_4$. The oil (0.86 g) obtained after evaporation of the solvent was characterized as the title compound on the basis of GC-MS analysis, in which peaks were seen at *m/z* 218 (<1%; M^+), 189 (1; $M^+ - 29$; loss of C_2H_5), 148 (14), 119 (13), 105 (21), 93 (60), 91 (56), and 79 (100), UV (λ_{max} [EtOH] 219 nm), and FT-IR, which gave bands at 1696 (aldehyde carbonyl), 1128, and 972 cm^{-1} (*E*-double bond).

*6(Z),9(Z),12(Z)-Pentadecatrienal (**5**).* A mixture of diisobutylaluminium hydride (20 mmol), hexamethylphosphoric triamide (10 mL; distilled fom calcium hydride), and CuI (200 mg) in 45 mL of dry THF was stirred under argon at 0°C for 20 min. Aldehyde **4** (0.86 g) in 15 mL of THF was added, and the mixture was stirred at 0°C for 1 h. Extraction with hexane provided **5** (0.66 g) as an essentially colorless oil showing *m/z* 220 (1%; M^+), 191 (1; $M^+ - 29$; loss of C_2H_5), 164 (6), 108 (19), and 79 (100).

*6(Z),9(Z),12(Z)-Pentadecatrienol (**6**).* The above-mentioned aldehyde **5** (0.66 g) was dissolved in 20 mL of methanol and cooled to 0°C. Sodium borohydride (500 mg) was added during 10 min under stirring. After further 20 min



SCHEME 1

at 23°C, water was added and the solution extracted with diethyl ether. Alcohol **6** thus obtained (0.63 g, purity ca. 95%) showed *m/z* 222 (4%; M^+), 166 (2), 108 (23), 93 (44), 91 (40), and 79 (100). FTIR (film) showed bands at 3351 (alcohol), 1456, 1055, and 714 cm^{-1}.

*3-Oxalinolenic acid (**7**).* To a solution of alcohol **6** (0.63 g) in toluene (26 mL) were added tetrabutylammonium chloride (187 mg), *tert*-butyl bromoacetate (2.85 g), and 8.75 g of a 50% (wt/vol) solution of NaOH in water. After addition of a crystal of BHT antioxidant and purging with argon, the mixture was stirred at room temp for 24 h. The *tert*-butyl ester of **7** obtained after extraction with diethyl ether was hydrolyzed by treatment with trifluoroacetic acid (4 mL) in dichloromethane (8 mL) at room temperature for 1 h. Purification by silicic acid column chromatography (elution with diethyl ether/hexane (3:7, vol/vol)) afforded **7** (0.47 g; purity, ca. 90%; yield from **1**, 32%). Final purification by RP-HPLC using solvent system A afforded >99% pure 3-oxalinolenic acid (0.18 g [0.64 mmol]; yield from **1**, 12%) as a colorless oil. Analysis of the methyl ester derivative by GC-MS using capillary columns of phenylmethylsilicone or Supelcowax showed single peaks having retention times corresponding to C-17.71 and C-20.58, respectively, and a mass spectrum showing *m/z* 294 (2%; M^+), 265 (1; $M^+ - 29$; loss of C_2H_5), 204 (2; $M^+ - 90$; loss of $OCH_2COOMe + H$), 175 (3), 108 (26), and 79 (100). The proton NMR (400 MHz, $CDCl_3$) spectrum showed signals at δ 0.97 ppm (t, J = 7.4, H-18), 1.35–1.47 (m, H-6 and H-7), 1.60–1.68 (m, H-5), 2.02–2.11 (m, H-8 and H-17), 2.78–2.83 (m, H-11 and H-14), 3.56 (t, J = 6.6, H-4), 4.10 (s, H-2), and 5.27–5.43 (m, H-9, H-10, H-12, H-13, H-15, H-16). That the *cis* configuration of the double bonds of **1** was retained in **7** was shown by the FTIR spectrum, which showed the absence of an absorption band at ca. 970 cm^{-1} typical for *trans* unsaturation.

*3-Oxa-13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid (**8**).* 3-Oxalinolenic acid **7** (2.4 mM) in 15 mL of sodium borate buffer pH 10.4 was stirred for 15 min at 0°C under oxygen atmosphere in the presence of soybean lipoxygenase-1 (170,000 units). The product was isolated by extraction with diethyl ether and subjected to open column silicic acid chromatography. Elution with diethyl ether-hexane (3:7, vol/vol) afforded the title compound in 90% yield showing UV absorption with λ_{max} (EtOH) 234 nm. An aliquot was subjected to catalytic hydrogenation using platinum catalyst to provide 3-oxa-13-hydroxystearic acid (mass spectrum of methyl ester/Me_3Si derivative: *m/z* 373 (1%; $M^+ - 15$), 317 (71; $M^+ - (CH_2)_4\text{-}CH_3$), 288 (39; rearrangement with loss of $OCH\text{-}(CH_2)_4\text{-}CH_3$), 173 (100; $Me_3SiO^+{=}CH\text{-}(CH_2)_4\text{-}CH_3$), 147 (61), and 73 (91; Me_3Si^+).

*3-Oxa-13(S)-hydroxy-9(Z),11(E),15(Z)-octadecatrienoic acid (**9**).* The hydroperoxide **8** (10 mg) was dissolved in 3 mL of ice-cold methanol and treated with 30 mg of $NaBH_4$ at 0°C for 15 min to provide the title compound in essentially quantitative yield. The UV spectrum showed λ_{max} (EtOH) = 233 nm and the mass spectrum of the methyl ester/Me_3Si derivative showed prominent ions at *m/z* 382 (1%; M^+), 323 (1; $M^+ - 59$; loss of COOMe), 313 (100; $M^+ - 69$; loss of $CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH_3$), 223 (11; 313-90), 133 (39), and 73 (99; Me_3Si^+).

*3-Oxa-13-oxo-9(Z),11(E),15(Z)-octadecatrienoic acid (**10**).* To hydroperoxide **8** (20 mg) was added an ice-cold mixture of acetic anhydride (0.25 mL) and pyridine (0.2 mL). After 13 min at 0°C, water (0.5 mL) was added, and the mixture was stirred for 30 min at 23°C. Purification by RP-HPLC using solvent system B provided **10** (effluent volume, 47.7–50.0 mL; 13.9 mg; 74% yield). The UV spectrum showed λ_{max} (EtOH) = 280 nm and the mass spectrum of the methyl ester showed prominent ions at *m/z* 308 (17%; M^+), 239 (59; $M^+ - 69$; loss of $CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH_3$), 149 (91; $[CH{=}CH\text{-}CH{=}CH\text{-}CO\text{-}CH_2\text{-}CH{=}CH\text{-}CH_2\text{-}CH_3]^+$), 121 (76), 107 (100), and 79 (91).

*3-Oxa-12-oxo-10,15(Z)-phytodienoic acid (**11**).* Product **8** (75 mg, 300 μM) was stirred at 23°C for 20 min with an acetone preparation of flaxseed (12) resuspended in the 100,000 × *g* supernatant of homogenate of potato tuber (13) as sources of allene oxide synthase and allene oxide cyclase, respectively. The product was subjected to RP-HPLC using solvent system C to provide **11** as a colorless oil (effluent volume, 59.5–62.8 mL; 15 mg, 21% yield). The UV spectrum showed λ_{max} (EtOH) = 220 nm, and the mass spectrum of the methyl ester showed prominent ions at *m/z* 308 (23%; M^+), 279 (12; $M^+ - 29$; loss of C_2H_5), 249 (7; $M^+ - 59$; loss of COOMe), 240 (34; $M^+ - 68$; β-cleavage with loss of $CH_2\text{-}CH{=}CH\text{-}C_2H_5$), 235 (16; $M^+ - 73$; loss of $CH_2\text{-}COOMe$), 218 (8; $M^+ - 90$; loss of $OH\text{-}CH_2\text{-}COOMe$), 149 (58; $M^+ - 159$; loss of $(CH_2)_5\text{-}O\text{-}CH_2\text{-}COOMe$), 107 (90), and 95 (100).

Incubations with 9-lipoxygenases. Recombinant maize 9-lipoxygenase was obtained following expression of the maize lipoxygenase gene (cssap92) in *E. coli* as described in the literature (14). The products formed after incubation of **7** (36 μM) with maize lipoxygenase in Tris buffer pH 7.0 at 23°C for 20 min under oxygen atmosphere were treated with sodium borohydride in methanol. The reduced material was derivatized by methyl-esterification and trimethylsilylation and analyzed by GC-MS. Incubations of **7** with tomato lipoxygenase (15) were carried out in potassium phosphate buffer pH 6.0 under oxygen, and aliquots of the mixture were removed for UV spectroscopic analysis.

Cloning and expression of Physcomitrella patens α-dioxygenase and Arabidopsis thaliana α-dioxygenase-1. Recombinant baculoviruses expressing *Arabidopsis thaliana* α-dioxygenase-1 (At-DOX1) or *Physcomitrella patens* α-dioxygenase (Pp-DOX) were generated using the Bac-to-Bac baculovirus expression system (InVitrogen, Paisley, UK). The generation of At-DOX1 baculovirus has been described previously (16). A 2.3-kb DNA fragment containing the entire cDNA for a *Physcomitrella patens* α-dioxygenase (*cf.* ref. 2) was obtained from an EST database (http://moss.nibb.ac.jp) (17). The Pp-DOX cDNA was excised from its host plasmid RAFL 12-25 with Sfi1, blunt-ended with T4 DNA polymerase, and ligated into the Stu1 restriction site of the pFastBac vector. Correct cloning of the insert was verified by restriction analysis. The recombinant plasmid was transferred

into DH10Bac *E. coli* cells containing the baculovirus shuttle vector bMON14272 and the helper plasmid pMON7124. Recombinant bacmid DNA was prepared from positive bacterial clones, and recombinant baculovirus was obtained by transfecting the bacmid into High Five insect cells according to manufacturer's instructions.

α-Dioxygenase expression was performed by infecting High Five insect cell cultures grown at 28°C in Tc100 medium supplemented with 10% fetal calf serum with the recombinant viruses. At 48 h (At-DOX1 expression) or 72 h (Pp-DOX expression) after infection, cells were collected by centrifugation (5 min, 3000 g), washed twice with Dulbecco's phosphate-buffered saline (pH 7.4), divided in aliquots and pelleted by centrifugation (5 min, 3000 g). Cell pellets were stored at –80°C until analysis. Total protein extracts of α-dioxygenase-expressing cells were separated by gel-electrophoresis and visualized by Coomassie Brilliant Blue staining.

Incubations with α-dioxygenases. High Five cells expressing Pp-DOX and At-DOX1 (190 and 470 μg total protein, respectively) were briefly sonicated on ice (3 × 0.3 s at 35 W) in 0.1 M Tris pH 7.4 and immediately incubated for 30 min at room temperature with **7** (100 μM) in a total volume of 3 mL of oxygenated 0.1 M Tris buffer at pH 7.4 containing 100 μM $CaCl_2$. The products were extracted with diethyl ether, derivatized, and analyzed by GC-MS.

Incubations with hydroperoxide-metabolizing enzymes. For studies of **8** as a precursor of cyclopentenone and ketol derivatives, an acetone powder preparation of allene oxide synthase from flaxseed (12) suspended in a 100,000 × *g* supernatant from potato homogenate containing allene oxide cyclase (13) were stirred with 300 μM **8** at 23°C for 20 min. The product was methyl-esterified and trimethylsilylated and analyzed by GC-MS. Metabolism of **8** by guava hydroperoxide lyase (18) and divinyl ether synthases from garlic (19) or *Ranunculus acris* (20) was studied under incubation conditions described as indicated. The reaction products isolated by extraction with diethyl ether were derivatized and analyzed by GC-MS.

Chemical, chromatographical, and instrumental methods. RP-HPLC was carried out with a column of Nucleosil C_{18} 100-7 (250 × 10 mm; Macherey-Nagel, Düren, Germany) and solvent systems of acetonitrile-water-acetic acid in proportions 60:40:0.005, by vol (solvent system A), 55:45:0.005, by vol (solvent system B), or 45:55:0.005, by vol (solvent system C) at a flow rate of 4 mL/min. The effluent was led to a Bischoff model DAD-100 diode-array detector (Bischoff Chromatography, Leonberg, Germany).

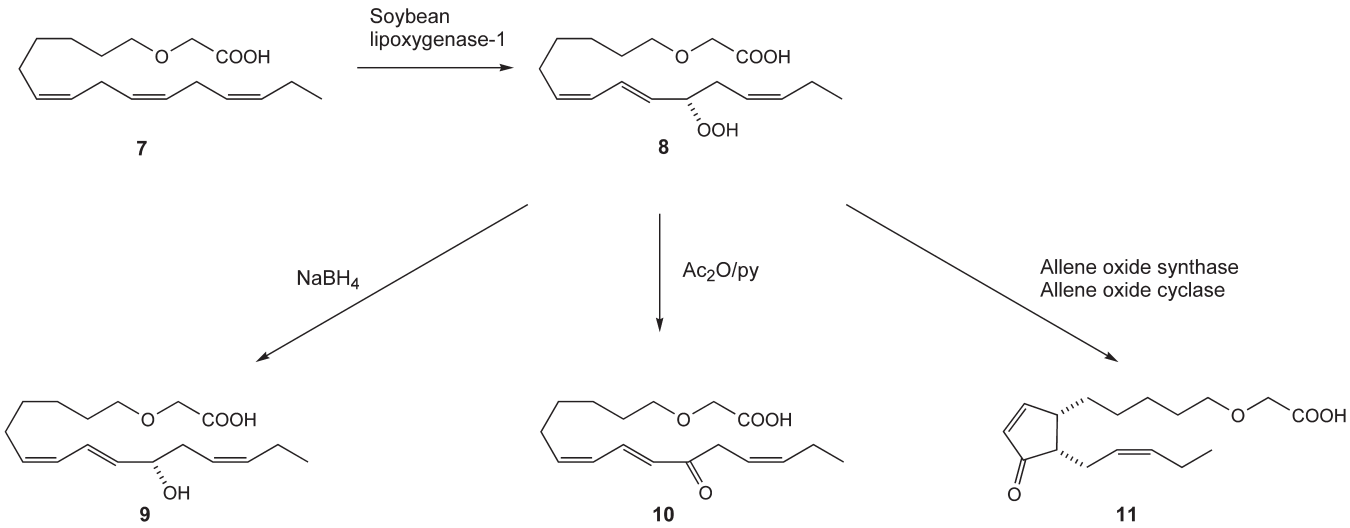
GLC with flame ionization detection was performed using a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph using either a methylsilicone capillary column (length 25 m, film thickness 0.33 μm) or a Supelcowax capillary column (length 30 m, film thickness 0.25 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas. C-values recorded during gas chromatography were calculated from diagrams constructed by plotting the retention times of standard saturated FAME on a logarithmic scale vs. the number of carbon atoms of the carbon chains on a linear scale. 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. FT-IR spectrometry was carried out using a PerkinElmer (Norwalk, CT) model 1650 FT-IR spectrophotometer. NMR spectra were recorded on a Varian Mercury plus spectrometer at 400 MHz. Samples were dissolved in deuteriochloroform, and chemical shifts are reported as δ values (ppm) and indirectly referenced to tetramethylsilane via the residual solvent signal ($CHCl_3$ in $CDCl_3$).

RESULTS AND DISCUSSION

3-Oxalinolenic acid (7). Synthesis of **7** was achieved by an eight-step sequence starting with 5(*Z*),8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosapentaenoic acid, a cheap and readily available natural product (Scheme 1). Iodolactonization and hydrolysis as originally described by Corey *et al*. (21) provided the 5,6-epoxide derivative of eicosapentaenoic acid. Further conversion by hydrolysis and periodate cleavage afforded tetraenal **3**, the β,γ-double bond of which was removed by isomerization into the α,β-position followed by selective reduction using diisobutyl aluminium hydride. Aldehyde **3** has been previously prepared by Skattebol and coworkers (10,11) and been further elaborated to sulfur- and oxygen-containing PUFA (10) as well as polyunsaturated trifluoromethyl ketones (11). Indeed aldehyde **3** and its isomerized and saturated analogs **4** and **5**, as well as other aldehydes derived from 5,8,11,14,17-eicosapentaenoic acid (22), appear to be useful starting materials for a range of ω-3 FA, and can also be used for the preparation of ω-3 FA isotopically labeled in the carboxyl end.

To the best of our knowledge, this study describes the first synthesis of 3-oxalinolenic acid, although the 3-oxa and 3-thia analogs of the related 6,9,12,15-octadecatetraenoic acid (stearidonic acid) have been prepared (10). 3-Oxa FA prepared in other studies are "unnatural" in the sense that their chain lengths consist of an odd number of atoms. Such FA include 3-oxa-6,9,12,15,18-heneicosapentaenoic acid (10) as well as 3-oxa-heneicosatrienoic acid, 3-oxa-heneicosatetraenoic acid, and 3-oxa-tricosatetraenoic acid (9,23,24). Among oxylipins, 3-oxa analogs of 10,11-dihydro-13-epi-12-oxo-PDA (25) and tetrahydrodicranenone B (26) have been prepared. Furthermore, β-oxidation–resistant 3-oxa analogs of the eicosanoid lipoxin A_4 have been synthesized (27).

3-Oxalinolenic acid was oxygenated by soybean lipoxygenase-1 to provide the corresponding 13(*S*)-hydroperoxide, and this compound could be further elaborated into 13-hydroxy or 13-oxo derivatives by reduction or dehydration, respectively (Scheme 2). On the other hand, no conversion of 3-oxalinolenic acid by tomato 9-lipoxygenase could be observed. In an earlier study, 2-hydroxylinolenic acid was found not to be a substrate for this enzyme (28). Taken together, these observations suggest that binding of the carboxyl end



SCHEME 2

plays an important role in the tomato 9-lipoxygenase-catalyzed oxygenation of FA. In previous studies, replacement of the carboxyl group of substrates for 9-lipoxygenases with nonionizable groups was found to affect the rate of oxygenation as well as the regiospecificity (29,30).

*Oxygenation of **7** by recombinant maize 9-lipoxygenase*. In contrast to tomato lipoxygenase, recombinant maize lipoxygenase catalyzed oxygenation of **7**, albeit at a slow rate (8% of the rate of linolenic acid oxygenation). The mass spectrum of the reduced and derivatized hydroperoxide product showed prominent ions at *m/z* 382 (1%; M^+), 313 (4; $M^+ - CH_2$-CH=CH-CH_2-CH_3), 292 (9; $M^+ - 90$), 223 (16; Me_3SiO^+=CH-(CH=CH)$_4$-CH_2-CH=CH-CH_2-CH_3), 151 (7), 133(14), 131 (11), 75 (100; $Me_2Si=O^+H$), and 73 (51; Me_3Si^+). In another experiment, the 3-oxa-9-hydroperoxide was converted into the saturated 3-oxa-9-hydroxystearate by sodium borohydride reduction followed by catalytic hydrogenation. The mass spectrum of the methyl ester/Me_3Si derivative of this compound showed prominent ions at *m/z* 373 (0.2%; $M^+ - CH_3$), 261 (53; Me_3SiO^+=CH-$(CH_2)_5$-O-CH_2-$COOCH_3$), 232 (29; $M^+ - 56$; rearrangement with loss of OHC-$(CH_2)_8$-CH_3), 229 (72; Me_3SiO^+=CH-$(CH_2)_8$-CH_3), 147 (39), and 73 (100; Me_3Si^+).

*Oxygenation of **7** by α-dioxygenases*. The effect of the 3-oxa substitution in **7** on the dioxygenation of the vicinal α-carbon by α-dioxygenases (2) was studied using recombinant enzymes from *Arabidopsis thaliana* and the moss *Physcomitrella patens*, At-DOX1 and Pp-DOX. These proteins were expressed in High Five insect cells following infection with recombinant baculoviruses containing the At-DOX1 or Pp-DOX pFastBac constructs, respectively (Fig. 1). The molecular masses of the expressed proteins were approximately 75 kDa and 70 kDa, respectively, in accordance with the size predicted for both proteins. The two α-dioxygenases were not expressed when insect cells were infected with baculovirus prepared from empty pFastBac vector.

The product **7** was oxygenated by At-DOX1 at a rate 25% of that of linolenic acid oxygenation. GC-MS analysis of the derivatized reaction products revealed comparable amounts of two main products identified as 6,9,12-pentadecatrienol and 6,9,12-pentadecatrienyl formate (Scheme 3). The mass spectrum of the first-mentioned compound was identical to that of authentic 6,9,12-pentadecatrienol (**6**), and the spectrum of the corresponding Me_3Si derivative showed ions at *m/z* 294 (3%; M^+), 175 (5), 129 (16; Me_3SiO^+=CH-CH=CH_2), 108 (39), 93 (48), 79 (100), and 73 (63; Me_3Si^+). As expected, catalytic hydrogenation afforded pentadecanol. The second product showed *m/z* 250 (4%; M^+), 221 (1; $M^+ - C_2H_5$), 194 (7), 135 (8), 93 (49), 79 (100), and 67 (67). Mild alkaline hydrolysis (0.3 M NaOH in 43% aqueous ethanol, 1

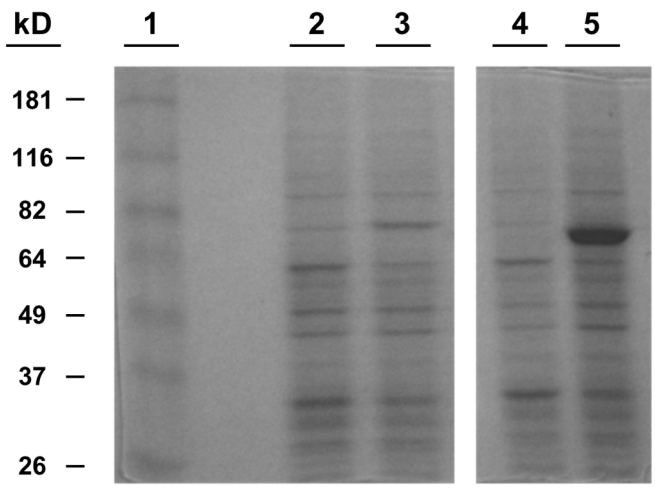
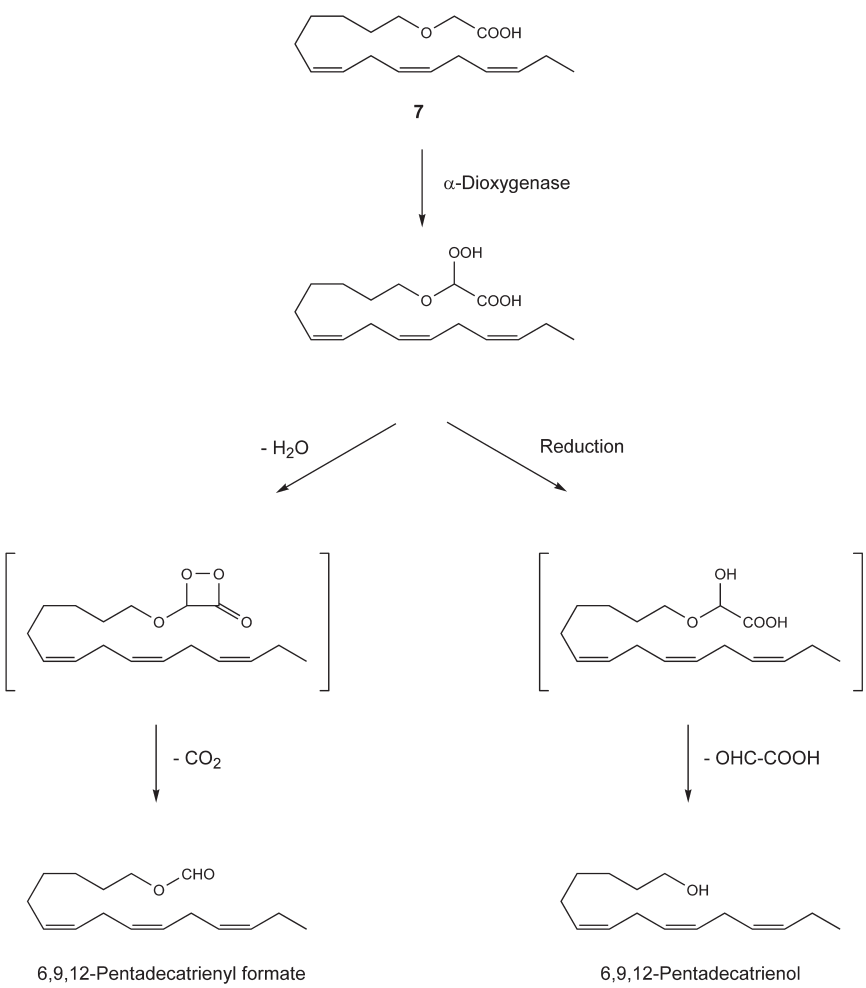


FIG. 1. SDS-polyacrylamide gel electrophoresis illustrating the expression of At-DOX1 and Pp-DOX in High Five insect cells infected with recombinant baculoviruses. Lane 1, molecular mass markers (indicated in kDa); lanes 2 and 4, cells infected with baculovirus without transposed gene; lane 3, cells infected with recombinant pFastBac/At-DOX1 baculovirus (48 h); lane 5, cells infected with recombinant pFastBac/Pp-DOX baculovirus (72 h). Each lane corresponds to protein lysates derived from approximately 125,000 cells.



SCHEME 3

h at 37°C) converted the product into 6,9,12-pentadecatrienol, indicating its identity with the formate ester of this alcohol. In previous work using pea α-dioxygenase, a 4-oxa FA (4-oxadodecanoic acid) was found not to serve as a substrate whereas a 5-oxa substituted FA (5-oxapentadecanoic acid) was efficiently oxygenated (31).

Pp-DOX was ca. 20% more active than At-DOX1 in oxygenating **7** and produced the same set of compounds. The mode of formation of these products can be rationalized in terms of the mechanism of α-dioxygenases and the known instability of 2-hydroperoxy FA (32). Thus, part of the α-dioxygenase product 3-oxa-2-hydroperoxylinolenic acid will decarboxylate to produce the formate ester of 6,9,12-pentadecatrienol, a process that probably takes place via a peroxylactone intermediate (Scheme 3). Another part will be reduced into the corresponding hydroxide prior to decarboxylation and produce a highly unstable hemiacetal that will rearrange spontaneously into 6,9,12-pentadecatrienol and glyoxylic acid.

*Conversion of **8** by allene oxide synthase/allene oxide cyclase*. GC-MS analysis of the derivatized products formed upon incubation of **8** (300 μM; 23°C, 20 min) with a preparation of flax allene oxide synthase and potato allene oxide cyclase showed the absence of remaining hydroperoxide and the presence of two main products formed in ca. 1:1 ratio. The earlier-eluting derivative was identified as the methyl ester of 3-oxa-12-oxo-PDA (**11**). The second derivative eluted just before the Me_3Si derivative of the methyl ester of 13-hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid. On the basis of its mass spectrum, which showed prominent ions at *m/z* 383 (4%; $M^+ - 15$; loss of CH_3), 339 (1; $M^+ - 59$; loss of COOMe), 329 (2; $M^+ - 69$; loss of CH_2-CH=CH-C_2H_5), 272 (50; $M^+ - 126$; rearrangement of Me_3Si with loss of CO(O)CH-CH_2-CH=CH-C_2H_5 or its equivalent), 249 (1; 339-90), 171 (100; Me_3SiO^+=CH-CH_2-CH=CH-C_2H_5), 129 (29; Me_3SiO^+=CH-CH=CH_2), and 73 (88; Me_3Si^+), it was tentatively identified as the Me_3Si derivative of the methyl ester of 3-oxa-13-hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid.

*Conversion of **8** by garlic and* Ranunculus *divinyl ether synthases*. The products obtained after incubation of **8** (300 μM; 23°C for 15 min) with whole homogenate preparations of garlic tuber (19) or leaves from *Ranunculus acris* (20) were methyl-esterified, trimethylsilylated, and subjected to GC-MS. This analysis showed the absence of derivatives of the methyl esters of **9** or **10** (on-column degradation products of

hydroperoxide, *cf*. ref. 33), thus indicating that hydroperoxide **8** had been completely consumed in the divinyl ether synthase reactions. Instead, major peaks eluting close to the divinyl ether etherolenic acid (garlic incubation) and ω-5(*Z*)-etherolenic acid (*Ranunculus* incubation) appeared. The identity of these materials with 3-oxa-etherolenic acid and 3-oxa-ω-5(*Z*)-etherolenic acid, respectively, was supported by mass spectrometry, which showed prominent ions at *m/z* 308 (72%; M^+), 292 (6), 241 (9), 189 (8), 171 (15), 133 (44), and 79 (100), and 308 (63%), 279 (3), 189 (7), 171 (14), 149 (28), and 79 (100), respectively. Although this experiment proved that **8** served as the substrate for garlic and *Ranunculus* types of divinyl ether synthases, no attempts were made to verify the double bonds of the resulting divinyl ethers with regard to position or configuration.

*Conversion of **8** by guava hydroperoxide lyase*. Incubation of **8** (300 μM; 23°C for 10 min) with a whole homogenate preparation of guava fruit (18) led to a product that was methyl-esterified, trimethylsilylated, and analyzed by GC-MS. As was the case with the divinyl ether synthase incubations, derivatives of the hydroperoxide degradation products **9** or **10** could not be detected, thus indicating a complete consumption of **8**. In another experiment, the incubation mixture was treated with an equal volume of 30 mM methoxyamine hydrochloride in methanol in order to facilitate detection of the expected aldehyde product. One major peak appeared showing the following prominent mass spectral ions: *m/z* 257 (2%; M^+), 226 (9; M^+ – OMe), 198 (6; M^+ – COOMe), 184 (30; M^+ – CH_2-COOMe), 93 (61), 80 (100), and 67 (99). The identity of the parent aldehyde with 3-oxa-12-oxo-9-dodecenoic acid was supported by an additional experiment, in which the incubation mixture was treated with an excess of sodium borohydride in methanol. The resulting major product was tentatively identified as 3-oxa-12-hydroxy-9-dodecenoic acid on the basis of its mass spectrum (methyl ester – Me_3Si derivative), which showed *m/z* 302 (0.3%; M^+), 272 (5), 212 (3; M^+ – Me_3SiOH), 147 (34), 103 (72; $Me_3SiO^+=CH_2$), and 73 (100; Me_3Si^+).

Conclusion. 3-Oxa-linolenic acid, a β-oxidation–resistant analog of linolenic acid, was synthesized in 10–15% yield starting with the easily accessible FA 5,8,11,14,17-eicosapentaenoic acid. 3-Oxalinolenic acid was found to be oxygenated by both lipoxygenases and α-dioxygenases. In agreement with a previous study using 3-oxa FA having acyl chains composed of an odd number of atoms (22), 3-oxalinolenic acid served as a good substrate for soybean lipoxygenase. The resulting 3-oxa-13-hydroperoxide was convertible chemically or by secondary enzymes into a range of 3-oxa oxylipins including 3-oxa-12-oxo-PDA. The biological activities of such oxylipin analogs are presently being explored.

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2,6-Hexadecadiynoic Acid and 2,6-Nonadecadiynoic Acid: Novel Synthesized Acetylenic Fatty Acids as Potent Antifungal Agents

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ABSTRACT: The hitherto unknown 2,6-hexadecadiynoic acid, 2,6-nonadecadiynoic acid, and 2,9-hexadecadiynoic acid were synthesized in two steps and in 11–18% overall yields starting from either 1,5-hexadiyne or 1,8-nonadiyne. Among all the compounds 2,6-hexadecadiynoic acid displayed the best overall antifungal activity against both the fluconazole-resistant *Candida albicans* strains ATCC 14053 and ATCC 60193, with a minimum inhibitory concentration (MIC of 11 μM), and against *Cryptococcus neoformans* ATCC 66031 (MIC < 5.7 μM). 2,9-Hexadecadiynoic acid did not display any significant cytotoxicity against the fluconazole-resistant *C. albicans* strains, but it showed fungitoxicity against *C. neoformans* ATCC 66031 with a MIC value of < 5.8 μM. Other FA, such as 2-hexadecynoic acid, 5-hexadecynoic acid, 9-hexadecynoic acid, and 6-nonadecynoic acid were also synthesized and their antifungal activities compared with those of the novel acetylenic FA. 2-Hexadecynoic acid, a known antifungal FA, exhibited the best antifungal activity (MIC = 9.4 μM) against the fluconazole-resistant *C. albicans* ATCC 14053 strain, but it showed a MIC value of only 100 μM against *C. albicans* ATCC 60193. 2,6-Hexadecadiynoic acid and 2-hexadecynoic acid also displayed a MIC of 140–145 μM toward *Mycobacterium tuberculosis* $H_{37}Rv$ in Middlebrook 7H12 medium. In conclusion, 2,6-hexadecadiynoic acid exhibited the best fungitoxicity profile compared with other analogues. This diynoic FA has the potential to be further evaluated for use in topical antifungal formulations.

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The 2-alkynoic FA have been known to be fungitoxic (1–2). The fungal activity of these compounds depends on the FA chain length and pH of the medium (1–2). The optimal chain lengths (between 8 and 16 carbons) have been established for the 2-alkynoic FA to exert maximum fungistatic effects (2). Among the 2-alkynoic FA 2-hexadecynoic acid has received the most attention for its antifungal, antimicrobial, and cytotoxic properties (3–5). The bioactivity of 2-hexadecynoic acid has been ascribed to its ability to inhibit the elongation of saturated and unsaturated FA as well as its potential to inhibit the FA acylation process, particularly triglyceride synthesis (4–5).

Other isomeric alkynoic FA, in particular the 6-alkynoic FA, are also fungistatic. For example, when an unsaturation is introduced between C-6 and C-7 in a fatty acyl chain, the FA displays good fungitoxicity. It is shown that 6-hexadecynoic acid is a good substrate for the enzyme myristoyl-CoA:protein *N*-myristoyltransferase from *Saccharomyces cerevisiae*, an important enzyme for fungal growth and a good target for antifungal therapy (6). Furthermore, 6-nonadecynoic acid, recently isolated from the roots of *Pentagonia gigantifolia*, is reported to be fungistatic against some fungal strains of fluconazole-resistant *Candida albicans* (7) as well as against *Cryptococcus neoformans* (8). *C. albicans* and *C. neoformans* are pathogenic fungi of concern in immunocompromised patients. 6-Alkenoic FA have also been evaluated. For example, 6-hexadecenoic acid is antimicrobial to gram-positive bacteria (MIC of 10–20 μg/mL) and blocks the adherence of *C. albicans* to porcine stratum corneum (9).

With this information at hand regarding the antifungal activities of 2-alkynoic and 6-alkynoic FA, we envisaged the possibility of combining in a single molecule the C-2 and C-6 ynoic functionalities by synthesizing 2,6-diynoic FA and evaluating their antifungal properties. It was expected that novel 2,6-diynoic FA would have a better antifungal profile than either of its parent 2-alkynoic or 6-alkynoic FA. The synthesis of 2,6-octadecadiynoic acid as a precursor to furan-containing FA has been previously reported by Lie Ken Jie *et al.* (10–11). However, to the best of our knowledge, the FA 2,6-hexadecadiynoic acid (**3a**) and 2,6-nonadecadiynoic acid (**3c**) are novel. We describe the synthesis of the dimethylene-interrupted diynoic FA **3a** and **3c**, as well as the synthesis of 2,9-hexadecadiynoic acid (**3b**). Comparative antifungal activities of the compounds indicate that **3a** has the best fungitoxicity profile among the studied compounds and the known 2-hexadecynoic acid.

EXPERIMENTAL PROCEDURES

Instrumentation. ^1H and ^{13}C NMR spectra were recorded on either a Bruker DPX-300 or a Bruker DRX-500 spectrometer. ^1H NMR chemical shifts are reported with respect to internal

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Abbreviations: AMB, amphotericin B; FLC, fluconazole; LORA, low oxygen recovery assay; MABA, microplate alamar blue assay; MIC, minimum inhibitory concentration; NCCLS, National Committee for Clinical Laboratory Standards; SDA, Sabouraud dextrose agar; SDB, Sabourand dextrose broth.

Me_4Si, and chemical shifts are given in parts per million (ppm) relative to $CDCl_3$ (77.0 ppm). Mass spectral data were acquired on a GC–MS (Hewlett-Packard 5972A MS ChemStation) instrument at 70 eV, equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) of polymethylsiloxane crosslinked with 5% phenyl methylpolysiloxane. Infrared spectra were recorded on a Nicolet 600 FT-IR spectrophotometer.

Microorganisms. C. albicans ATCC 60193, *C. albicans* ATCC 14053, and *C. neoformans* ATCC 66031 were obtained from American Type Culture Collection (Manassas, VA). Stock cultures were kept on Sabouraud dextrose agar (SDA; Becton-Dickinson and Co., Sparks, MD). Subcultures were prepared on SDA at 35–37°C. Suspension cultures were prepared by inoculation of single colonies in 7 mL of normal saline solution. Prior to preparation of susceptibility assays, yeast cells were resuspended in normal saline to yield a transmittance of 73–75% at 530 nm, which provided an equivalent concentration of 10^6 cells/mL. The medium was Sabourand dextrose broth (SDB; Becton Dickinson and Co.).

Chemicals and antifungal agents. Amphotericin B (AMB) was purchased from Acros Organics (Geel, Belgium), and was kept as a 5-mM stock in DMSO at 0°C and used within 1 wk of preparation. Fluconazole (FLC) was purchased from Medisa Inc. (New York, NY), or was provided from Vera Laboratories Ltd. (Hyderabad, India), and was kept as a 20-mM stock solution at 0°C. Working dilutions were made in SDB medium. Higher concentrations of compounds were used for those with weak antifungal activities. The final maximum concentration of DMSO in the assays was 5% (vol/vol). DMSO was not inhibitory to the organisms tested.

Susceptibility testing. Microdilutions for control experiments with *C. albicans* and *C. neoformans* were performed according to the modified method of the National Committee for Clinical Laboratory Standards (NCCLS) as described by Galgiani (12) and according to the more recent NCCLS M27-A microdilution methods as described previously (13–14). Dilutions were prepared in 0.1 mL of SDB; the inocula were 10^4 cells of either *C. albicans* or *C. neoformans*. The tubes were incubated for 24–48 h at 36 ± 1°C, and turbidity was read visually. MIC were calculated in comparison with growth control as the lowest concentration that showed inhibition for AMB, FLC, and the test compounds.

General procedure for the monoalkylation of the diynes. To a stirred solution of the diyne (8.3–12.8 mmol) in dry THF (20–25 mL), *n*-Buli (2.5 M, 7.5–11.5 mmol) in dry hexane (3.0–5.0 mL) was added dropwise while keeping the temperature at –78°C. After 45 min, hexamethylphosphoramide (HMPA) (3.0–5.0 mL) and the bromoalkane (7.7–11.5 mmol) were added dropwise to the reaction mixture while maintaining the temperature at –78°C. After 24 h, the reaction mixture was worked up by pouring into a large volume of water, and extracting with diethyl ether (2 × 20 mL). The organic layer was washed with brine (1 × 20 mL) before drying ($MgSO_4$). Filtration, rotoevaporation of the solvent, and fractional distillation afforded the monoalkylated diynes **2a**–**2c** in 21–47% yields after purification by Kugel-Rohr distillation (60–100°C and 3 mm Hg) of the impurities.

(i) 1,8-Pentadecadiyne (***2b***). 1,8-Pentadecadiyne (**2b**) was obtained in a 21% yield as a viscous oil from the reaction of 1.22 mL of 1,8-nonadiyne (1.00 g, 8.32 mmol) and 1.16 mL of 1-bromohexane (1.37 g, 8.32 mmol) according to the general procedure described above. IR (neat) ν_{max} 3311, 2932, 2859, 2118, 1463, 1332, 1192, 1100, 629 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.19 (2H, *dt*, *J* = 2.6 and 6.9 Hz, H-10), 2.13 (4H, m, H-3, H-7), 1.93 (1H, *t*, *J* = 2.6 Hz, H-1), 1.57–1.24 (14H, m, CH_2), 0.88 (3H, *t*, *J* = 7.0 Hz, H-15); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 84.53 (*s*, C-2), 80.48 (*s*), 79.84 (*s*), 68.15 (*d*, C-1), 31.36 (*t*, C-13), 29.10 (*t*), 28.61 (*t*), 28.53 (*t*), 28.04 (*t*), 27.95 (*t*), 22.56 (*t*, C-14), 18.73 (*t*), 18.69 (*t*), 18.33 (*t*), 14.04 (*q*, C-15); GC–MS *m/z* (relative intensity) M^+ 204 (0.1), 189 (1), 175 (4), 161 (4), 147 (14), 133 (28), 119 (32), 107 (11), 105 (54), 95 (13), 91 ($C_7H_7^+$, 100), 81 (30), 79 (61), 67 (51), 55 (31).

(*ii*) *1,5-Pentadecadiyne* (***2a***). 1,5-Pentadecadiyne (**2a**) was obtained in a 40% yield as a viscous oil from the reaction of 2.5 mL of 1,5-hexadiyne (1.00 g, 12.80 mmol) and 2.2 mL of 1-bromononane (2.38 g, 11.50 mmol) according to the general procedure described above. IR (neat) ν_{max} 3313, 2926, 2855, 2122, 1466, 1338, 1257, 635 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.38 (4H, *m*, H-3, H-4), 2.14 (2H, *brt*, *J* = 7.0 Hz, H-7), 2.00 (1H, *t*, *J* = 2.0 Hz, H-1), 1.52–1.27 (14H, *m*, CH_2), 0.88 (3H, *t*, *J* = 6.9 Hz, H-15); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 83.11 (*s*, C-2), 81.61 (*s*), 78.09 (*s*), 68.94 (*d*, C-1), 31.89 (*t*, C-13), 29.50 (*t*), 29.28 (*t*), 29.16 (*t*), 28.97 (*t*), 28.83 (*t*), 22.67 (*t*, C-14), 19.18 (*t*), 18.93 (*t*), 18.71 (*t*), 14.09 (*q*, C-15); GC–MS *m/z* (relative intensity) M^+ 204 (0.1), 161 (1), 147 (3), 133 (13), 119 (19), 109 (6), 105 (31), 95 (16), 91 ($C_7H_7^+$, 100), 81 (22), 79 (31), 67 (32), 65 (20), 55 (29).

(*iii*) *1,5-Octadecadiyne* (***2c***). 1,5-Octadecadiyne (**2c**) was obtained as a viscous oil in a 47% yield from the reaction of 2.5 mL of 1,5-hexadiyne (2.00 g, 12.80 mmol) and 1.8 mL of 1-bromododecane (1.87 g, 7.70 mmol) according to the general procedure described above. IR (neat) ν_{max} 3313, 2925, 2854, 2122, 1466, 1338, 1257, 635 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.38 (4H, *m*, H-3, H-4), 2.14 (2H, *brt*, *J* = 6.9 Hz, H-7), 2.00 (1H, *m*, H-1), 1.55–1.26 (20H, *m*, CH_2), 0.88 (3H, *t*, *J* = 6.8 Hz, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 83.11 (*s*, C-2), 81.62 (*s*), 78.09 (*s*), 68.93 (*d*, C-1), 31.92 (*t*, C-16), 29.67 (*t*), 29.64 (*t*), 29.63 (*t*), 29.55 (*t*), 29.35 (*t*), 29.16 (*t*), 28.97 (*t*), 28.83 (*t*), 22.68 (*t*, C-17), 19.18 (*t*), 18.93 (*t*), 18.71 (*t*), 14.10 (*q*, C-18); GC–MS *m/z* (relative intensity) M^+ 246 (0.1), 175 (1), 161 (2), 147 (8), 133 (27), 119 (27), 105 (38), 95 (16), 91 ($C_7H_7^+$, 100), 81 (20), 79 (35), 67 (28), 65 (24), 55 (29).

General procedure for the carboxylation of the alkylated diynes. To a stirred solution of the alkylated diynes (**2a**–**2c**, 1.7–4.6 mmol) in dry THF (17–46 mL), *n*-Buli (2.5 M, 2.6–7.2 mmol) in dry hexane (1–3 mL) was added dropwise while keeping the temperature approximately at –78°C. After 45 min the reaction mixture was treated with dry CO_2 (by passing the CO_2 through sulfuric acid) and left stirring for 24 h. The reaction mixture was then worked up by pouring into a large volume of a saturated solution of ammonium chloride followed by extraction with diethyl ether (3 × 20 mL). Rotoevaporation of the solvent afforded the diynoic acids **3a**–**3c** in 28–52% yields

after purification by Kugel-Rohr distillation (60–100°C and 3 mm Hg) of the impurities. The higher yield (52%) was obtained for 2,9-hexadecadiynoic acid (**3b**).

*(i) 2,9-Hexadecadiynoic acid (**3b**).* 2,9-Hexadecadiynoic acid (**3b**) was obtained as an oil in a 52% yield from the reaction of 1,8-pentadecadiyne (0.35 g, 1.70 mmol) and excess dry CO_2 (by passing the CO_2 through sulfuric acid) according to the general procedure described above. IR (neat) ν_{max} 3400–2900 (*br*), 2932, 2859, 2238, 1688, 1462, 1410, 1332, 1279, 1076 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.36 (2H, *t*, *J* = 7.1 Hz, H-4), 2.14 (4H, *m*, H-8, H-11), 1.62–1.23 (14H, *m*, CH_2), 0.88 (3H, *t*, *J* = 7.0 Hz, H-16); ^{13}C NMR ($CDCl_3$,125 MHz) δ 157.08 (*s*, C-1), 92.01 (*s*, C-3), 81.24 (*s*), 80.71 (*s*), 72.69 (*s*, C-2), 31.35 (*t*, C-14), 29.08 (*t*), 28.54 (*t*), 28.43 (*t*), 27.96 (*t*), 26.98 (*t*), 22.56 (*t*, C-15), 18.72 (*t*), 18.69 (*t*), 18.55 (*t*), 14.04 (*q*, C-16).

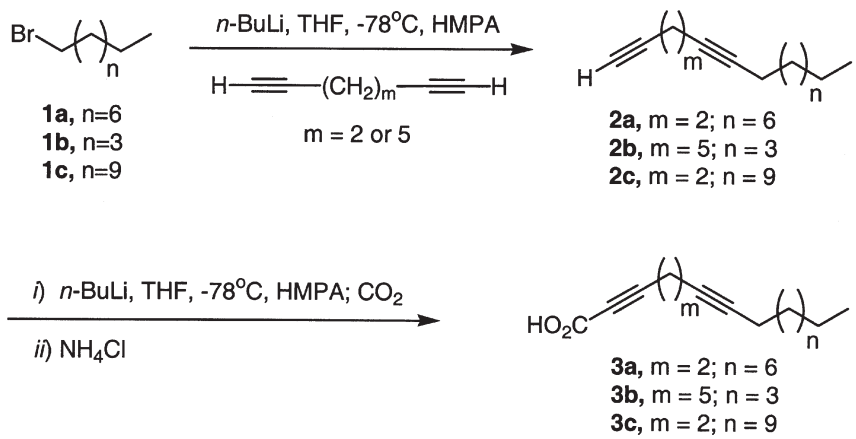
*(ii) 2,6-Hexadecadiynoic acid (**3a**).* 2,6-Hexadecadiynoic acid (**3a**) was obtained as a white solid in a 45% yield from the reaction of 1,5-pentadecadiyne (0.94 g, 4.60 mmol) and excess dry CO_2 (by passing the CO_2 through sulfuric acid) according to the general procedure described above. M.p. 48–50°C, IR (neat) ν_{max} 3400–2900 (*br*), 2953, 2915, 2848, 2246, 1674, 1469, 1425, 1300, 1276, 1075, 890, 608 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz,) δ 2.54 (2H, *brt*, *J* = 7.3 Hz, H-4), 2.44 (2H, *m*, H-5), 2.14 (2H, *tt*, *J* = 2.2 and 7.1 Hz, H-8), 1.47 (2H, *m*, H-9), 1.40–1.27 (12H, *m*, CH_2), 0.88 (3H, *t*, *J* = 6.9 Hz, H-16); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 156.58 (*s*, C-1), 90.10 (*s*, C-3), 82.44 (*s*), 77.10 (*s*), 73.14 (*s*, C-2), 31.83 (*t*, C-14), 29.48 (*t*), 29.29 (*t*), 29.15 (*t*), 28.86 (*t*), 28.83 (*t*), 22.67 (*t*, C-15), 19.51 (*t*, C-4), 18.67 (*t*, C-8), 17.94 (*t*, C-5), 14.08 (*q*, C-16). Anal. calcd. for $C_{16}H_{24}O_2$: C, 77.38%; H, 9.74%. Found: C, 76.17%; H, 10.19%.

*(iii) 2,6-Nonadecadiynoic acid (**3c**).* 2,6-Nonadecadiynoic acid (**3c**) was obtained as a white solid in a 28% yield from the reaction of 1,5-octadecadiyne (0.89 g, 3.60 mmol) and excess dry CO_2 (by bubbling the CO_2 through sulfuric acid) according to the general procedure described above. M.p. 62–64°C, IR (neat) ν_{max} 3400–2900 (*br*), 2953, 2916, 2848, 2245, 1671, 1422, 1280, 912, 720 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 2.54 (2H, *brt*, *J* = 7.4 Hz, H-4), 2.44 (2H, *m*, H-5), 2.14 (2H, *tt*, *J* = 2.3 and 7.1 Hz, H-8), 1.47 (2H, *m*, H-9), 1.38–1.25 (18H, *m*, CH_2), 0.88 (3H, *t*, *J* = 6.9 Hz, H-19); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.96 (*s*, C-1), 90.33 (*s*, C-3), 82.44 (*s*), 77.21 (*s*), 73.06 (*s*, C-2), 31.91 (*t*, C-17), 29.64 (*t*), 29.53 (*t*), 29.35 (*t*), 29.14 (*t*), 28.84 (*t*), 22.68 (*t*, C-18), 19.49 (*t*, C-4), 18.66 (*t*, C-8), 17.91 (*t*, C-5), 14.11 (*q*, C-19). Anal. calcd. for $C_{19}H_{30}O_2$: C, 78.57%; H, 10.41%. Found: C, 77.14%; H, 10.40%.

RESULTS AND DISCUSSION

Seven acetylenic FA were synthesized and evaluated for antifungal activities against *C. albicans* and *C. neoformans*. The acetylenic FA 2-hexadecynoic acid, 5-hexadecynoic acid, 9-hexadecynoic acid, and 6-nonadecynoic acid were synthesized according to the previously described procedures (1,8,15). Despite the fact that 2,6-octadecadiynoic acid has been previously synthesized (10–11), there are no reported syntheses for acids **3a**–**3c**. Therefore, a simple synthetic methodology for the preparation of novel FA **3a**–**3c** was developed based on two consecutive acetylide coupling reactions.

The synthesis of the dimethylene-interrupted 2,6-hexadecadiynoic acid (**3a**) and 2,6-nonadecadiynoic acid (**3c**) was conveniently accomplished using 1,5-hexadiyne (50% in pentane) as the starting material (Scheme 1). In the preparation of **3a**, 1,5-hexadiyne was coupled with 1-bromononane using *n*-BuLi in THF/HMPA at –78°C, which afforded 1,5-pentadecadiyne (**2a**) in a 40% yield. Likewise, for the synthesis of acid **3c**, 1,5-hexadiyne was coupled with 1-bromododecane using the same acetylide coupling conditions, which afforded 1,5-octadecadiyne (**2c**) in a 47% yield after final purification (Scheme 1). Coupling of the lithium acetylides of 1,5-pentadecadiyne (**2a**) or 1,5-octadecadiyne (**2c**) with carbon dioxide and subsequent protonation with ammonium chloride afforded 2,6-hexadecadiynoic acid (**3a**) or 2,6-nonadecadiynoic acid (**3c**) in 28–45% yields after final purification (Scheme 1). The overall yields for these two two-step syntheses ranged between 13 and 18%.



SCHEME 1

TABLE 1
Antifungal Activity Against *Candida albicans* (SDB) and *Cryptococcus neoformans* (SDB) at 35–37°C after 24–48 h[a]

	MIC (μM)		
Compound	*C. albicans* ATCC 14053	*C. albicans* ATCC 60193	*C. neoformans* ATCC 66031
2-Hexadecynoic	9.4	100.3	<6.3
5-Hexadecynoic	7,845	10,460	61.3
9-Hexadecynoic	8,260	11,014	10.8
6-Nonadecynoic	6,672	8,896	<4.3
2,6-Hexadecadiynoic (**3a**)	11.5	11.5	<5.7
2,9-Hexadecadiynoic (**3b**)	4,469	2,235	<5.8
2,6-Nonadecadiynoic (**3c**)	20.0	80.2	<5.0
FLC	>2,000	>1,000	<0.9
AMB	<0.3	<0.3	<0.3
DMSO	>5,000	>5,000	>5,000

[a]The results are the average of three separate experiments. SDB, Sabourand dextrose broth; FLC, fluconazole; AMB, amphotericin B.

The preparation of 2,9-hexadecadiynoic acid (**3b**) followed a similar synthetic strategy, with the exception that this synthesis started with the 1,8-nonadiyne. The diyne was coupled with 1-bromohexane using again *n*-BuLi in THF/HMPA at –78°C, which afforded 1,8-pentadecadiyne (**2b**) in a 21% yield after final purification. Final coupling of the lithium acetylide of 1,8-pentadecadiyne (**2b**) with carbon dioxide and subsequent protonation with ammonium chloride afforded the desired 2,9-hexadecadiynoic acid (**3b**) in a 52% yield after final purification (Scheme 1). The overall yield for this two-step synthesis was 11%.

The antifungal activity of the synthesized acetylenic FA against *Candida albicans* strains ATCC 14053 and ATCC 60193, and *Cryptococcus neoformans* ATCC 66031 in SDB were determined using a modified method of the National Committee for Clinical Laboratory Standards (NCCLS) as described by Galgiani and the more recent NCCLS M27-A microdilution methods as described previously (12–13) (Table 1). AMB and FLC were used as positive controls. Among the monoynoic FA, 2-hexadecynoic acid exhibited the best MIC values against FLC-resistant *C. albicans* ATCC 14053 and ATCC 60193 strains; these MIC values were 9.4 and 100 μM, respectively. However, as we have previously reported (8), 6-nonadecynoic acid showed the best MIC value (<4.3 μM) against *C. neoformans* ATCC 66031, but it was not very effective against the FLC-resistant *C. albicans* strains studied herein (8). Therefore, introduction of a triple bond at either C-2 or C-6 in the alkyl chain was effective in increasing the fungitoxicity of the FA. However, a triple bond at either C-5 or C-9 in the alkyl chain was not particularly effective in increasing the fungitoxicity of the FA.

Among the studied diynoic FA, 2,6-hexadecadiynoic acid (**3a**) displayed the best MIC values against both FLC-resistant *C. albicans* strains, ATCC 14053 and ATCC 60193, with MIC values of 11.5 μM. However, 2,6-nonadecadiynoic acid (**3c**) displayed the best MIC value (<5.0 μM) among the diynoic acids against *C. neoformans* ATCC 66031, but it displayed modest activity against the studied FLC-resistant *C. albicans* strains, with MIC values between 20 and 80 μM (Table 1). A comparison of the bioactivity of **3a** with that of the 2-hexadecynoic acid reveals that introduction of a second triple bond at C-6 increases the fungitoxicity of the acid toward *C. albicans* ATCC 60193 (MIC = 11.5 μM) approximately 9-fold compared with 2-hexadecynoic acid (MIC = 100 μM). Therefore, the diynoic acid **3a** has the potential of displaying a broader antifungal profile than the parent 2-hexadecynoic acid.

The acid 2,9-hexadecadiynoic acid (**3b**) was also studied, and it was not particularly effective against the FLC-resistant *C. albicans* strains studied herein, but it displayed fungitoxicity against *C. neoformans* ATCC 66031 with a MIC of < 5.8 μM. A comparison of the bioactivity of **3b** with that of the 9-hexadecynoic acid reveals that introduction of a second triple bond at C-2 increases the fungitoxicity of the parent 9-hexadecynoic acid against the three fungal strains studied, but the bioactivity of **3b** was still poor as compared with that of either **3a** or **3c**.

In order to determine the selectivity of the 2,6-hexadecadiynoic acid (**3a**) toward fungi (eukaryotic pathogens) vs. bacteria we chose to explore the antimycobacterial activity of both **3a** and 2-hexadecynoic acid toward *Mycobacterium tuberculosis* $H_{37}Rv$ following a procedure previously described (16). We decided on *M. tuberculosis* because there is a recent literature report indicating that 2-hexadecynoic acid can be toxic (MIC ~ 10–20 μM in Sauton's medium) to mycobacteria by the accumulation of two metabolites, specifically, 3-ketohexadecanoic acid, which blocks FA biosynthesis, and 3-hexadecynoic acid, an inhibitor of FA β-oxidation

TABLE 2
MIC of 2,6-Hexadecadiynoic Acid and 2-Hexadecynoic Acid Against *Mycobacterium tuberculosis* $H_{37}Rv$[a]

Compound	MIC (μM)	
	MABA	LORA
2,6-Hexadecadiynoic (**3a**)	145	>300
2-Hexadecynoic	141	>300
Rifampin	0.08	1.5

[a]MABA, microplate alamar blue assay; LORA, low oxygen recovery assay.

(17). 2-Hexadecynoic acid, therefore, can inhibit mycolic acid biosynthesis, FA biosynthesis, and FA degradation (β-oxidation) pathways of importance for mycobacteria (17).

Our antimycobacterial results are shown in Table 2. Two assays were used to study the antimycobacterial activity of the acetylenic FA: the microplate alamar blue assay (MABA) and the low oxygen recovery assay (LORA). The MABA is the "normal" MIC determination against replicating *M. tuberculosis* under aerobic conditions for 8 d. On the other hand, the LORA utilizes *M. tuberculosis* under anaerobic nonreplicating conditions for 11 d. As can be seen from Table 2, both acetylenic FA displayed similar MIC of around 140–145 μM (in Middlebrook 7H12 medium) against *M. tuberculosis* using the MABA assay, but also both acids were not inhibitory to nonreplicating and persistent *M. tuberculosis*, as determined by the LORA assay (16). From these experiments we can conclude that the additional Δ6 triple bond in **3a** does not help in increasing its antimycobacterial activity as compared with the 2-hexadecynoic acid, a fact that supports its postulated mechanism of action (17). However, it is evident that **3a** is a better antifungal agent than antimycobacterial agent due to its specificity toward fungal cells, because the additional Δ6 triple bond in **3a** makes a difference in the antifungal activity.

With respect to the toxicity of the 2-alkynoic acids it was recently demonstrated that 2-alkynoic acids could be used specifically against bacteria without toxicity to their host due to the fact that the FASI complex in microsomal systems is not inhibited by the 2-alkynoic acids (17). In addition, it was previously reported that despite the fact that 2-hexadecynoic acid inhibits the growth of HeLa cells, the simultaneous addition of palmitic acid to the culture medium reverses the growth inhibition observed in HeLa cells, thus implying that toxicity would not occur in animals consuming a normal diet (3).

Further experiments are required to determine the mechanism of antifungal activities by these novel 2,6-diynoic FA. One possible mechanism of action for the 2,6-diynoic FA is isomerization to a 2,3-allene (as the CoA derivative) and subsequent inhibition of the biosynthesis of the fungal FA as shown for the 2-hexadecynoic acid in intact animals (4). Likewise, also analogous to the suspected mechanism of action for 6-nonadecynoic acid, the 2,6-diynoic FA could also inhibit sphingolipid biosynthesis in these fungi (7). Both mechanisms of lipid biosynthesis inhibition could also be operative, thus explaining the enhanced activity of **3a**. Compound **3a** has the potential for further evaluation in topical formulations for the treatment of fungal infections, as an antifungal in wound dressings, or as a preservative in skin and hair care products.

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Differential Biohydrogenation and Isomerization of [U-^{13}C]Oleic and [1-^{13}C]Oleic Acids by Mixed Ruminal Microbes

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ABSTRACT: The additional mass associated with ^{13}C in metabolic tracers may interfere with their metabolism. The comparative isomerization and biohydrogenation of oleic, [1-^{13}C]oleic, and [U-^{13}C]oleic acids by mixed ruminal microbes was used to evaluate this effect. The percent of stearic, *cis*-14 and -15, and *trans*-9 to -16 18:1 originating from oleic acid was decreased for [U-^{13}C]oleic acid compared with [1-^{13}C]oleic acid. Conversely, microbial utilization of [U-^{13}C]oleic acid resulted in more of the ^{13}C label in *cis*-9 18:1 compared with [1-^{13}C]oleic acid (53.7 vs. 40.1%). The isomerization and biohydrogenation of oleic acid by ruminal microbes is affected by the mass of the labeled tracer.

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Metabolic studies often use chemical tracers to evaluate *in vivo* events. A common method to create metabolic tracers is to replace an atom of the tracee molecule with a radioactive or stable isotope of that atom. These types of isotopes are less abundant in nature and are therefore easily distinguished from the isotope that is naturally more abundant (1). A summary of several studies has established that the use of tracer and higher doses of stable isotopes *in vivo* present no identifiable risk to human subjects (2). Therefore, *in vivo* measurements of FA metabolism may be obtained from the analysis of ^{13}C-labeled FA. Today, the use of stable isotopes to evaluate FA metabolism in humans is not uncommon (3,4). However, there is concern that not all isotopically labeled molecules are utilized equally. For example, different isotopically labeled glucose molecules are metabolized to varying extents (5,6). Furthermore, position and number of ^{14}C in labeled FA has been found to impact the extent of β-oxidation (7).

Previous research (8) has illustrated that [1-^{13}C]oleic acid is isomerized and biohydrogenated by ruminal microbes. Using these results as a model, it was hypothesized that there was no difference in the utilization of unlabeled oleic, [1-^{13}C]oleic, and [U-^{13}C]oleic acids by ruminal microbes despite the increased mass of the ^{13}C-labeled oleic acid molecules. Mixed ruminal microbes are necessary to carry out all possible isomerization and biohydrogenation processes (9). Additionally, ruminal microbes do not catabolize, elongate, or desaturate external FA to any great extent (10). Therefore, this model will only focus on differences in the isomerization and biohydrogenation of the treatment FA of differing masses without altering the isotope distribution in other metabolic pools.

EXPERIMENTAL PROCEDURES

Reagent grade oleic acid (97%) was purchased from Acros Organics (Geel, Belgium). [1-^{13}C]Oleic acid (99%) and [U-^{13}C]oleic acid (99%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All solvents were HPLC or GC grade. Dimethyl disulfide (DMDS) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Anhydrous ethyl ether, iodine, and sodium thiosulfate were purchased from Fisher Scientific (Pittsburgh, PA).

Microbial biohydrogenation of oleic acid was studied in cultures of microbes taken from the rumen of cattle as previously described (8). Cultures containing unlabeled, [1-^{13}C]-labeled, or [U-^{13}C]-labeled oleic acid were run in triplicate at 39°C under anaerobic conditions. Samples (5 mL) were taken from each culture at 0 and 48 h and immediately frozen. The samples were freeze-dried and then methylated using a two-step sodium methoxide and methanolic HCl procedure (11).

DMDS adducts of the FAME were prepared (8). The FAME and DMDS were analyzed by GC-MS (Agilent Technologies 6890N GC equipped with a 30 m × 0.25 mm with 0.2-μm film DB-225ms capillary column [Agilent J&W Scientific] and a 5973 inert series quadrupole mass-selective detector controlled by MSD ChemStation software [D.01.02.16] in the scan mode). For the FAME samples, column temperature was programmed from 50°C to 220°C at a rate of 6°C/min and held for 15 min. For the DMDS samples, column temperature was programmed from 195°C to 230°C at a rate of 0.5°C/min. Additionally, FAME were analyzed on a GC to determine the FA profile (12).

In order to measure the change in the ratio of ^{13}C to ^{12}C, the tracer (^{13}C) to tracee (^{12}C) ratio (TTR) for each FA was calculated from the mass abundance of the ^{13}C ($M + n$) and ^{12}C (M)

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Abbreviations: DMDS, dimethyl disulfide; E, enrichment; TTR, tracer to tracee ratio.

fragments using the equation TTR = $(M + n)/M$, where n is the number of ^{13}C atoms present in the fragment (1). In order to account for the natural levels of ^{13}C, the average TTR of samples taken from the unlabeled oleic acid cultures was subtracted from the TTR of samples from the ^{13}C-labeled oleic acid cultures, and the result was then adjusted for spectrum skew (1).

ANOVA using the GLM procedure of SAS (v. 9.1, SAS Institute, Inc., Cary, NC) was utilized to evaluate the ^{13}C enrichments at 0 and 48 h. Also, in an effort to evaluate the response to isotopic label, the degrees of freedom were partitioned into linear contrasts. All results are expressed as least square means. Additionally, ANOVA with the main effect of time on the percentage of each FA originating from oleic acid at 48 h and the distribution of the ^{13}C label at 48 h was evaluated for the [1-^{13}C]oleic and [U-^{13}C]oleic acid treatments. Least square means for each FA by treatment were analyzed for differences using Fisher's least significant difference with significance declared at $P \leq 0.05$.

RESULTS

Oleic acid was isomerized to a variety of *cis*- and *trans*-18:1 isomers and biohydrogenated to stearic acid (18:0) at the end of 48 h (Fig. 1). The other sources of oleic acid and its isomerization and biohydrogenation products in the microbial cultures (from the rumen inoculum and hay substrate) were minimal, and only linoleic (18:2 n-6) and linolenic (18:3 n-3) acids were considered in the data analysis. As expected, no enrichment was detected at either 0 or 48 h in the unlabeled oleic acid treatment. Excluding the minor contamination of [1-^{13}C]*trans*-9 18:1 (<1%) in the [1-^{13}C]oleic acid treatment and [U-^{13}C]*cis*-11 18:1 (5.5%) in the [U-^{13}C]oleic acid treatment, only oleic acid was enriched at 0 h in these two treatments (data not shown). Enrichment of *cis*-11 18:1 only occurred in the [U-^{13}C]oleic acid treatment. Therefore, only the change in weight percentage of *cis*-11 18:1 was evaluated.

The percentage of 18:0 and 18:1 isomers originating from oleic acid at 48 h was also evaluated. Excluding *cis*-9 and *cis*-

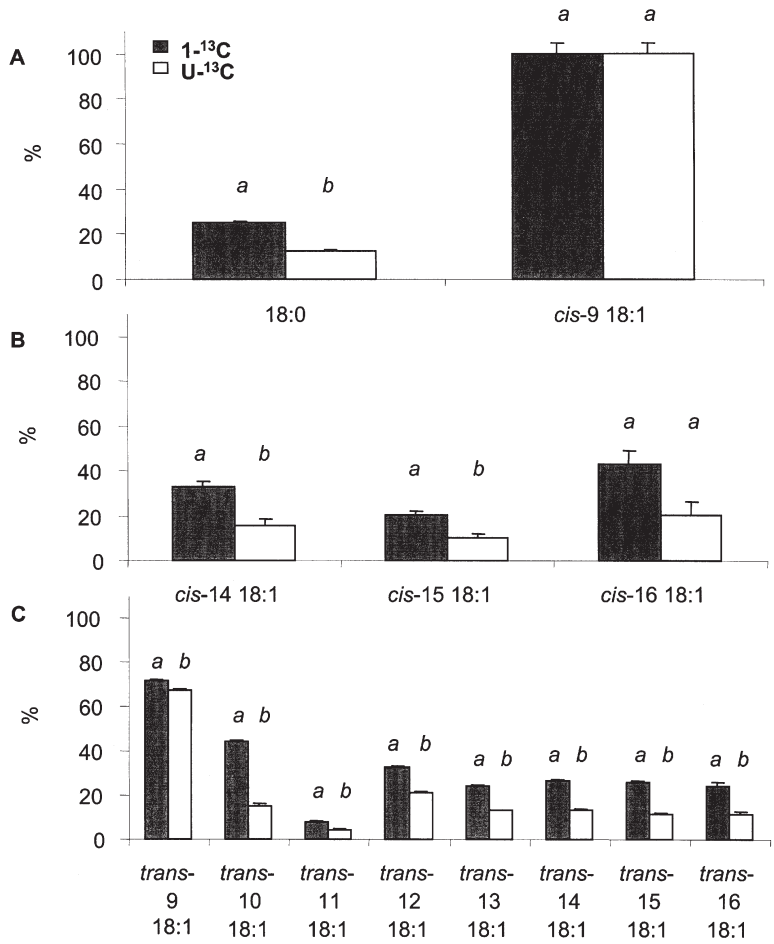

FIG. 1. Percent of (A) stearic and oleic, (B) *cis*-18:1, and (C) *trans*-18:1 FA originating from stably labeled oleic acid in microbial cultures. Determination occurred 48 h after [1-^{13}C]oleic (1-^{13}C) or [U-^{13}C]oleic (U-^{13}C) acid was added (25 mg per culture) to flasks. Values are mean ± SEM ($n = 3$). The SEM for 18:0 and *trans*-13 18:1 are too small to be viewed on figure. Columns with different letters differ ($P < 0.05$) within each FA variable.

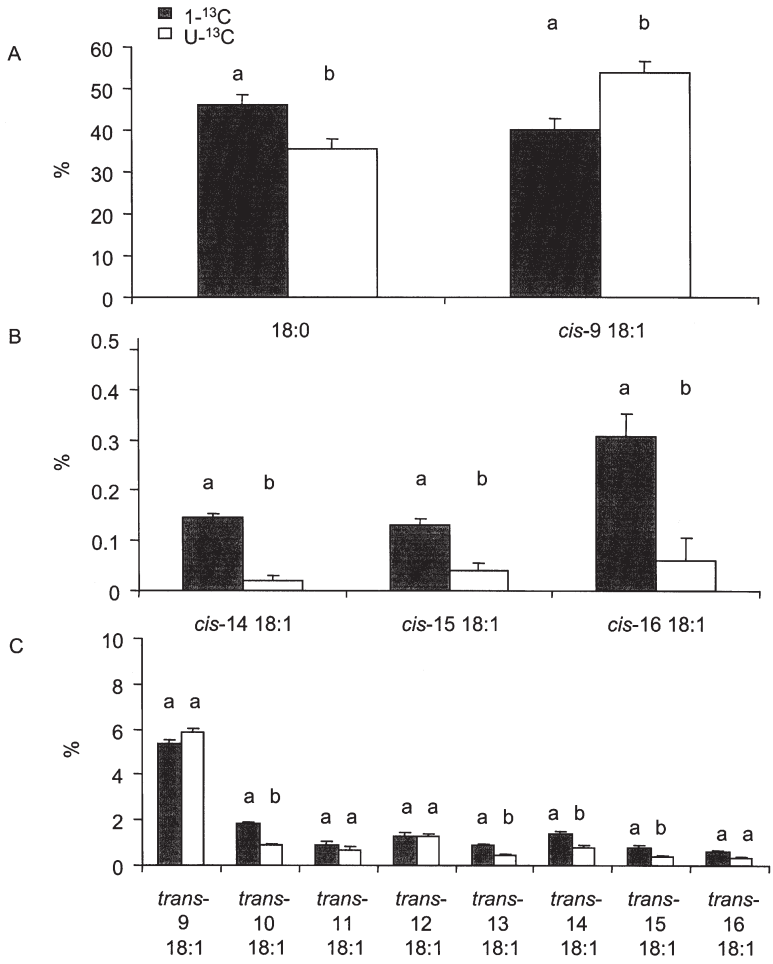

FIG. 2. Relative distribution of ^{13}C label from oleic acid in (A) stearic and oleic, (B) *cis*-18:1, and (C) *trans*-18:1 FA in microbial cultures 48 h after addition of [1-^{13}C]oleic (1-^{13}C) or [U-^{13}C]oleic (U-^{13}C) acid (25 mg per culture) to flasks. Values are mean ± SEM (n = 3). Columns with different letters differ ($P < 0.05$) within each FA variable.

16 18:1 isomers, there was a lower percentage of all other FA originating from oleic acid at 48 h for the [U-^{13}C]oleic acid treatment compared with the [1-^{13}C]oleic acid treatment (Fig. 1). Furthermore, when the overall distribution of the ^{13}C label was examined, treatment differences were observed (Fig. 2). A greater percentage of label remained in *cis*-9 18:1 in the [U-^{13}C]oleic acid treatment, whereas more of the label was detected in 18:0 and 18:1 isomers (*cis*-14, *cis*-15, *cis*-16, *trans*-10, *trans*-13, *trans*-14, and *trans*-15) in the [1-^{13}C]oleic acid treatment.

At 48 h, the weight percentage of 18:0 and most *cis*- and *trans*-18:1 FA increased (Table 1). The starting proportions of 18:0 in the cultures were variable among cultures; but no significant effect of isotopic label was detected. The *cis*-9 18:1 (oleic acid) weight percentage decreased over time; however, this was only a tendency when the singly and uniformly labeled oleic acid treatments were compared. The variation resulting from the contamination of the [U-^{13}C]oleic acid treatment with [U-^{13}C] *cis*-11 18:1 resulted in no differences observed when the unlabeled treatment was compared with the isotopically labeled treatments. However, when the isotopically labeled treatments were compared, differences in the concentration of [U-^{13}C]*cis*-11 18:1 were detected. Furthermore, differences due to addition of isotopic label and number of isotopes resulted in changes in the concentrations of several of the *cis*- and *trans*-18:1 FA (Table 1).

DISCUSSION

In our experiment, oleic acid was converted to stearic acid and a multitude of *cis*- and *trans*-18:1 isomers as previously described (8). Despite contamination of the ^{13}C FA sources, the formation of *trans*-18:1 monoenes from oleic acid is in agreement with previous research (8). It is unknown whether the *cis*- and *trans*-18:1 monoenes that are formed from oleic acid are intermediates or if they are end products of microbial manipulation of oleic acid. However, *trans*-9 18:1 was extensively intra-isomerized to other *cis*- and *trans*-18:1 and hydrogenated to stearic acid (13). Therefore, isomerization of oleic acid may be followed by further isomerization or hydrogenation to stearic acid.

TABLE 1
Proportions of Stearic Acid (18:0) and 18:1 FA Detected at 0 and 48 h when Oleic, [1-^{13}C]Oleic, or [U-^{13}C]Oleic Acids Were Added (25 mg) to Microbial Cultures[a]

	Treatment								
	Oleic		[1-^{13}C]Oleic		[U-^{13}C]Oleic			P^b	
Fatty acid	0 h	48 h	0 h	48 h	0 h	48 h	SE	1	2
18:0	6.57	65.23	10.39	63.19	11.95	66.23	1.39	NS	NS
18:1 Isomers									
cis-9	84.08	12.32	81.07	13.67	73.61	12.75	1.87	NS	0.07
cis-11	0	2.06	0	2.01	4.24	2.53	0.86	NS	0.03
cis-12	0	0.34	0.21	0.27	0	0.27	0.04	NS	0.02
cis-13	0.81	0.16	0.53	0.08	0.80	0.09	0.04	0.03	0.02
cis-14	0	0.02	0.02	0.15	0	0.03	0.01	<0.01	<0.01
cis-15	0	0.02	0.06	0.22	0.04	0.09	0.01	<0.01	<0.01
cis-16	0	0.07	0.04	0.25	0	0.67	0.02	0.06	<0.01
trans-5	0	1.17	0	0.92	0.07	0.84	0.03	<0.01	NS
trans-6	0	0.62	0	0.55	0	0.44	0.01	<0.01	<0.01
trans-7/8	0	3.63	0	3.09	0	2.66	0.11	<0.01	0.10
trans-9	0.34	2.32	0.47	2.56	0.52	2.05	0.06	NS	<0.01
trans-10	0	1.61	0	1.45	0	1.40	0.04	0.03	NS
trans-11	0.76	3.61	0.91	3.76	0.95	3.76	0.10	NS	NS
trans-12	0.06	1.62	0.17	1.36	0	1.39	0.10	NS	NS
trans-13	0.06	0.89	0.16	1.23	0.07	0.84	0.08	NS	0.02
trans-14	0.01	1.60	0.10	1.80	0	1.38	0.12	NS	0.07
trans-15	0.04	0.54	0.08	1.05	0	0.73	0.07	0.03	0.04
trans-16	0.51	0.38	0.08	0.86	0.20	0.70	0.21	NS	NS
18:2 n-6	2.89	0.92	2.63	0.76	3.58	0.87	0.06	NS	<0.01
18:3 n-3	3.88	0.87	3.11	0.76	3.97	0.90	0.10	0.09	<0.01

[a]The quantity of each FA is given as mg/100 mg of the FA presented. Values are the mean of three replicates at each time.
[b]1, oleic acid vs. the mean of [1-^{13}C]oleic acid and [U-^{13}C]oleic acid treatments; 2, [1-^{13}C]oleic acid vs. [U-^{13}C]oleic acid treatment; NS, nonsignificant.

Uniform, multiple, or single isotopically labeled FA are used to examine FA metabolism *in vivo* in humans and animals. Early milk fat researchers used isotopically labeled FA to examine the contribution of dietary FA to milk fat (14,15). Additionally, ^{2}H-labeled FA have been used to investigate the flow of FA through various plasma lipid classes in lactating women (16) and to determine the impact of FA position and structure of dietary triglycerides on absorption (17). However, there is limited research on the comparative utilization of stably labeled lipid and FA tracers of varying mass. When [23,24,25,26,27-^{13}C]cholesterol, [26,26,26,27,27,27-^{2}H]cholesterol, and [4-^{14}C]cholesterol were given intravenously and orally to healthy adult men and women, no differences in metabolism were observed (18). Similarly, when sample dilution correction factors were applied, no differences were detected in utilization of [17,17,18,18,18-^{2}H]linolenic, [U-^{13}C]linolenic, [17,17,18,18,18-^{2}H]linoleic, and [U-^{13}C]linoleic acids after simultaneous oral administration to rats (19). However, in isolated mitochondria, β-oxidation of [16-^{14}C]palmitic and [U-^{14}C]palmitic acids was reduced compared with [1-^{14}C]palmitic acid (7). Using mixed microbial cultures, slight differences were observed between the metabolism of unlabeled and isotopically labeled oleic acid. Furthermore, differences were also observed when singly or uniformly labeled oleic acid were compared (Table 1). These alterations in metabolism of isotopically labeled oleic acid by mixed ruminal microbes may be simply due to increases in the mass of the molecule or possibly due to some other isotope effect.

Despite differences in the extent of isomerization and biohydrogenation between the [1-^{13}C]oleic and [U-^{13}C]oleic acids, the same FA were enriched with ^{13}C. Additionally, the spectral shift associated with the use of [U-^{13}C]oleic acid is detected with less difficulty, as the mass fragments produced by MS analysis do not occur in the unlabeled oleic acid, but those produced by [1-^{13}C]oleic acid do occur. For example, the major spectral fragments 217 and 218 are used to identify FAME-DMDS derivatives of oleic and [1-^{13}C]oleic acids, respectively. Both of these fragments occur in the mass spectra of the FAME-DMDS derivative of unlabeled oleic acid. However, the major spectral fragment of [U-^{13}C]oleic acid is 226, which is a negligible fragment of oleic and [1-^{13}C]oleic acid. Furthermore, the detection of isotope discrimination may be trivial in mechanistic evaluations due to the relatively small differences observed. In the current study the entire FA pool is saturated with the labeled isotope, resulting in enrichment values of ^{13}C based on TTR calculations >100%. This large incorporation of labeled mass into the FA pool is not considered a tracer dose. For example, average *in vivo* measures of enrichment based on the TTR calculation in one study were <10% (12) when tracer doses of the labeled FA are used. Thus, there would be a considerable shift in the isotopic pool size being measured in the current study compared with other *in vivo* experiments.

The use of isotopically labeled FA has proven beneficial to research in lipid metabolism. The development of stable isotope tracers is necessary to safely study *in vivo* FA utilization in humans and other animals. However, researchers should consider the possible alterations in substrate utilization that result from some isotopically labeled compounds.

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The Inhibitory Action of Long-Chain Fatty Acids on the DNA Binding Activity of p53

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ABSTRACT: The *in vitro* relationship between human p53 DNA binding domain (p53 DBD) and FA was investigated. We found that saturated and monounsaturated long-chain FA inhibited the double-stranded DNA (dsDNA) binding activity of p53 DBD. The strongest inhibitors of saturated and unsaturated FA were docosanoic acid (22:0) and *cis*-12-heneicosenoic acid (21:1n-9), respectively. *n*-Octadecane, *trans*-unsaturated FA, and FAME had no influence on the binding activity of p53 DBD, showing that the FA structures such as one or no double bond of *cis* configuration, hydrocarbon chain of length C_{20} to C_{22}, and free carboxyl groups are important for the inhibition. The inhibitory effect of the R248A mutant of p53 DBD by saturated FA was as strong as that for wild-type p53 DBD. On the other hand, the inhibition of dsDNA binding activity of the same mutant by the *cis*-configuration of monounsaturated FA was weaker than that for the wild type. These results suggest that R248 in p53 DBD is important for binding to monounsaturated FA. This is the first report that long-chain FA act as a dsDNA binding inhibitor of p53, and it could be considered that FA in the cell membrane might regulate the activity of p53 for cell division, cell-cycle checkpoint, and tumor suppression.

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The tumor suppressor protein p53 (393 amino acids) is a transcription factor that functions to maintain the integrity of the genome (1). On its induction in response to DNA damage, p53 promotes cell-cycle arrest in the G1 phase (2) and apoptosis if DNA repair is not possible (3). The p53 gene encodes a protein with a central DNA binding domain (DBD) comprising residues 102–292, flanked by an N-terminal transactivation domain and a C-terminal tetramerization domain (4). The crystal structure of the core domain (i.e., p53 DBD) bound to DNA has been determined (5). The structure of the DNA binding domain consists of a large β-sandwich that acts as a scaffold for three loop-based elements that contact the DNA (5). Importantly, the residues most frequently mutated in cancers are all at or near the protein-DNA interface, and over two-thirds of the missense mutations are within the DNA binding loops (6). Although it is clear that tumorigenic p53-DBD mutations act by diminishing the DNA binding activity of p53 (7,8), the mechanisms are not fully understood.

We have been screening the products of microbial fermentation for compounds that inhibit the double-stranded DNA (dsDNA) binding activity of human p53-DBD, because the inhibitor can elucidate the structure and function of p53. Recently, we found an inhibitor from a basidiomycete (*Ganoderma lucidum*), a well-known FA, stearic acid (18:0). Subsequently, we investigated the effects of many commercially available FA on the activity of p53. We found that several FA interact with p53-DBD and strongly suppress the activity. In this paper, we discuss the effects of FA on the activity of p53. This study of FA may help to clarify the structure and function of p53-DBD.

EXPERIMENTAL PROCEDURES

Fatty acids. The following FA were purchased from Sigma (St. Louis, MO): saturated FA including hexadecanoic acid (palmitic acid, 16:0), octadecanoic acid (stearic acid, 18:0), nonadecanoic acid (19:0), eicosanoic acid (arachidic acid, 20:0), heneicosanoic acid (21:0), docosanoic acid (behenic acid, 22:0), tricosanoic acid (23:0), tetracosanoic acid (lignoceric acid, 24:0), hexacosanoic acid (26:0), methyl octadecanoate (methyl-18:0), and 12-hydroxy-octadecanoic acid (12-hydroxy-18:0), and unsaturated FA including *cis*-9-hexadecenoic acid (palmitoleic acid, 16:1n-7), *cis*-9-octadecenoic acid (oleic acid, 18:1n-9), *cis*-10-nonadecenoic acid (19:1n-9), *cis*-11-eicosenoic acid (gadoleic acid, 20:1n-9), *cis*-13-docosenoic acid (erucic acid, 22:1n-9), *cis*-14-tricosenoic acid (23:1n-9), *cis*-15-tetracosenoic acid (24:1n-9), *trans*-9-octadecenoic acid (elaidic acid, 18:1n-9), and 12-hydroxy-*cis*-9-octadecenoic acid (12-hydroxy-18:1n-9). *n*-Octadecane was also purchased from Sigma. *cis*-9,12-Octadecedienoic acid (linoleic acid, 18:2n-6), *cis*-9,12,15-octadecatrienoic acid (α-linolenic acid, 18:3n-3), *cis*-6,9,12,15-octadecatetraenoic acid (parinaric acid, 18:4n-3), and *cis*-12-heneicosenoic acid (21:1n-9) were obtained from Nu-Chek Prep Inc. (Elysian, MN). To avoid oxidation, these compounds were dissolved in DMSO and stored under nitrogen.

Materials. Supercoiled pBR322 plasmid dsDNA was obtained from Takara Bio Inc. (Kyoto, Japan). All other reagents

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Abbreviations: DBD, DNA binding domain; dsDNA, double-stranded DNA; NP-40, Nonidet P-40.

were of analytical grade and purchased from Nacalai Tesque (Kyoto, Japan).

Expression and purification of the human p53 DBD. The human p53-DNA binding domain (p53 DBD) gene (residues 92–292) was cloned into pET28b between *Nde*I and *Xho*I sites. The histidine-tagged protein was overexpressed in *Escherichia coli* strain BL21(DE3) harboring the expression plasmid constructed in our laboratory. The expression vector of the mutant protein (i.e., R248A) was constructed by a QuikChange site-directed mutagenesis kit (Stratagene, Ladolla, CA) and fully sequenced. The mutant protein of p53 DBD was overexpressed in *Escherichia coli* strain BL21(DE3) harboring the expression plasmid pET28b. After Ni-NTA column (Qiagen, Hilden, Germany) purification, following the procedure recommended by the manufacturer, the mutant proteins were purified on a heparin column (GE Health Care Bio-Sciences, Little Chalfant, UK) by elution with a concentration gradient of 0–1 M potassium chloride.

Gel mobility shift assay. The gel mobility shift assay was carried out as described by Casas-Finet *et al*. (9). The standard binding mixture (a final volume of 20 µl) contained 20 mM Tris-HCl, pH 7.5, 10% glycerol, 10% DMSO, 1 mM EDTA, 0.20 pmol pBR322 dsDNA (i.e., 870 pmol nucleotide), and 30.0 pmol (1.50 µM) human p53 DBD. The mixture was incubated for 20 min on ice. Samples were run on a 1.0% agarose gel in 0.1 M Tris-acetate, pH 8.3, containing 5 mM EDTA at 100 V for 1 h. The agarose gels were stained with ethidium bromide and dsDNA was visualized on a UV transilluminator. The gel mobility shift length of pBR322 dsDNA was measured with computer software (Zero-D scan, version 1.0, M&S Instruments Trading, Tokyo, Japan).

Surface plasmon resonance. Human p53 DBD and FA binding analyses were performed using a Biosensor BIAcore instrument (BIACORER X) (BIAcore, Uppsala, Sweden). CM5 research grade sensor chips (BIAcore) were used. All buffers were filtered before use. Human p53 DBD (25.5 kDa, 480 µg/ml, 30 µL, i.e., 565 pmol) in coupling buffer (10 mM sodium acetate, pH 4.7) was injected over a CM5 sensor chip at 20 µL/min to capture the protein to the carboxymethyl dextran matrix of the chip by NHS/EDC coupling reaction (60 µL of mix) as described (10). Unreacted *N*-hydroxysuccinimide ester groups were inactivated using 1 M ethanolamine-HCl (pH 8.0). This reaction immobilized about 5,000 response units (RU) of the protein. Binding analysis of FA was performed in running buffer including FA (5 mM potassium phosphate buffer (pH 7.0) and 10% DMSO) at a flow rate of 20 mL/min at 25°C. Kinetic parameters were determined using BIA evaluation 3.1 software.

Structural models of the complex. Molecular docking of human p53 DBD and FA was performed using the Affinity program within the Insight II software (Accelrys, San Diego, CA). The coordinates of human p53 DBD (Protein Data Bank (PDB) ID: 1TUP) were obtained from PDB. The initial position of FA was determined based on the result of the dsDNA gel mobility shift assay with the mutant p53 DBD and the protein surface of hydrophobicty. The calculation used a CVFF force field in the Discovery program (Accelrys).

RESULTS AND DISCUSSION

Analysis of the dsDNA binding activity of human p53 DBD. We first established the assay method of the dsDNA binding activity of human p53 DBD, which is the central region of p53, using agarose gel electrophoresis. As shown in Fig. 1A, 30.0 pmol of p53 DBD bound to pBR322 dsDNA, and the complex of p53DBD and dsDNA was performed, and the dsDNA stained with ethidium bromide was shifted to the

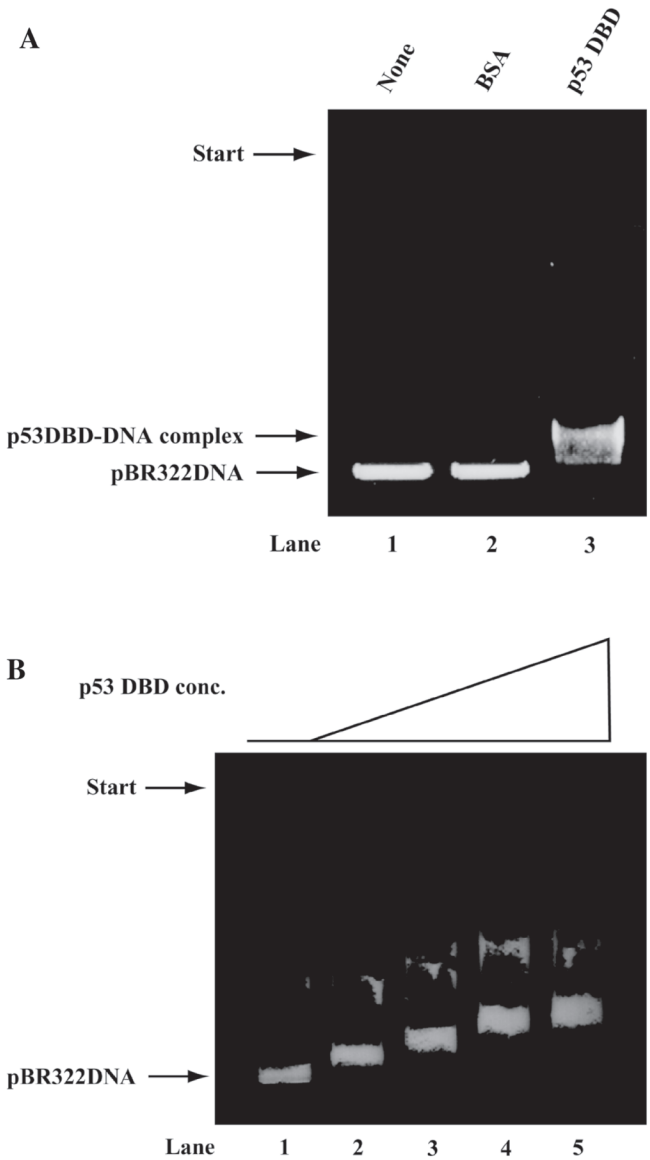

FIG. 1. The dsDNA binding activity of human p53 DBD. (A) Gel mobility shift assay of binding between pBR322 plasmid dsDNA (0.20 pmol; 870 pmol of nucleotide) and BSA (30.0 pmol, lane 2) or p53 DBD (30.0 pmol, lane 3). Lane 1 contained no proteins. (B) Gel mobility shift assay of binding between pBR322 dsDNA and p53 DBD. Lanes 1–5, purified p53 DBD at amounts of 0, 15.0, 30.0, 45.0, and 60.0 pmol, respectively. A photograph of an ethidium bromide-stained gel is shown.

upper side (lane 3). Because the same amount of BSA (bovine serum albumin) instead of p53 DBD did not shift at all (lane 2), dsDNA binding activity could occur selectively to a specific site on p53 DBD. The activity of p53 DBD was dose-dependent, and the distance of the stained and shifted band could be proportional to the dsDNA binding activity (Fig. 1B). We decided the activity by the shifted distances of the band, and 30.0 pmol of p53 DBD (i.e., 1.50 μM, lane 3) was used in the experiments below in this study.

Inhibitory effects of FA on the activity of human p53 DBD. As described earlier, the inhibitor of dsDNA binding activity of human p53 DBD was screened from the natural sources, and we found that a major FA, stearic acid (18:0), from a basidiomycete (*Ganoderma lucidum*), inhibited the activity. Since stearic acid is a C_{18} FA, inhibition of the activity of p53 DBD with commercially purchased C_{18} FA and related compounds was investigated (Fig. 2A). The 1.20 nmol (i.e., 60.0 μM) of the compound in the reaction mixture was 40-fold higher than that of the p53 DBD molecule (i.e., the amount of p53 DBD was 30.0 pmol). Stearic acid and oleic acid (*cis*-18:1n-9), which have a free-carboxyl group, inhibited the activity of p53 DBD, and the inhibitory effect of oleic acid was stronger than that of stearic acid. Other compounds such as *trans*-9-octadecenoic acid (elaidic acid: *trans*-18:1n-9), 12-hydroxy-octadecanoic acid (12-hydroxy-18:0), 12-hydroxy-*cis*-9-octadecenoic acid (12-hydroxy-18:19) and methyl octadecanoate (methyl-18:0) had no influence on the activity of p53 DBD, and the inhibition was less than 50%. Therefore, *trans*-configuration of the double bond, hydroxyl group and ester of the carboxyl group in C_{18} FA suggested the suppression of the inhibition.

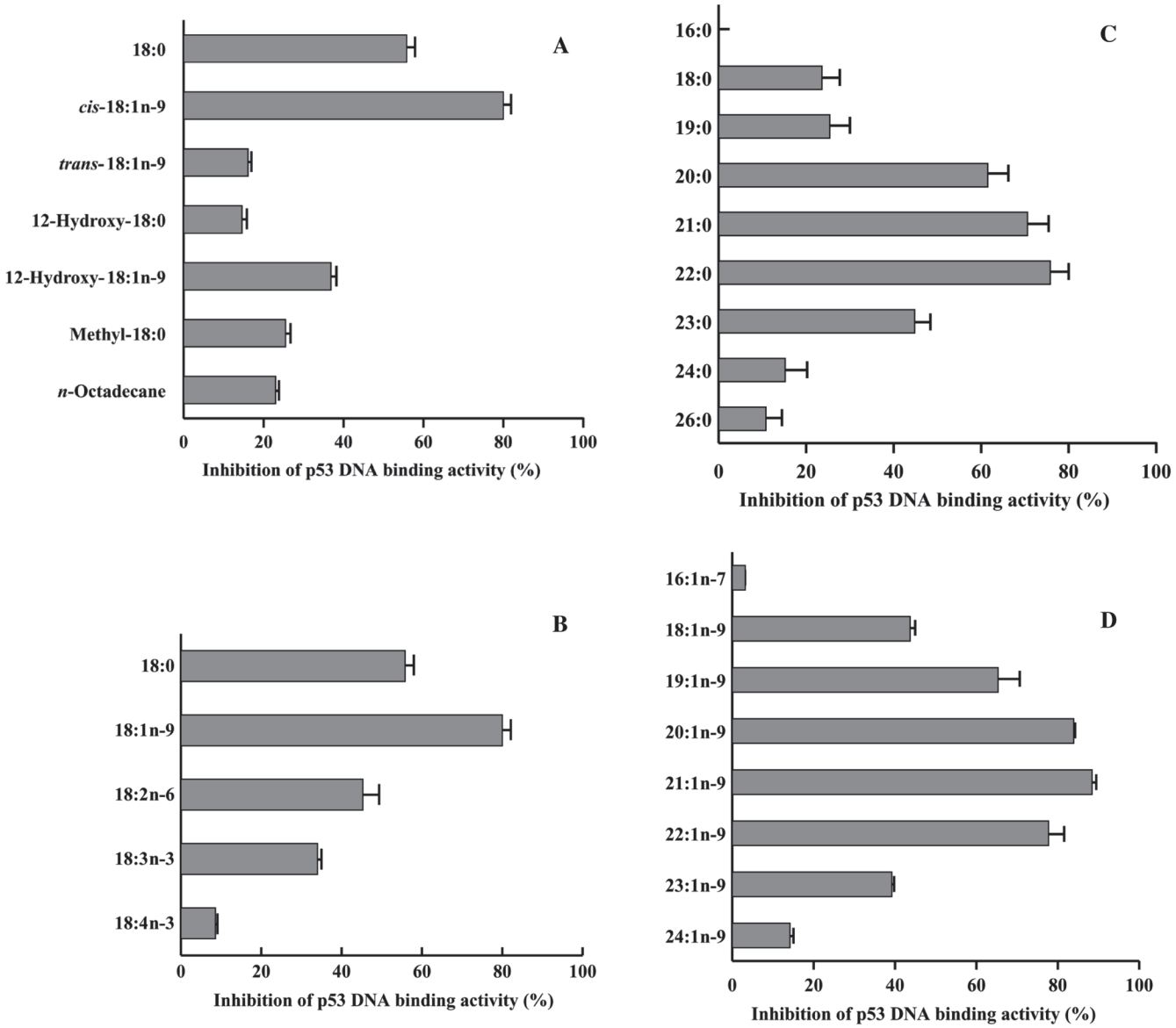

FIG. 2. Inhibitory effect of FA (1.20 nmol each) on the dsDNA binding activities of human p53 DBD. (A) C_{18} FA and related compounds, (B) C_{18} PUFA, (C) $C_{16\text{-}26}$ saturated FA, and (D) monounsaturated FA of *cis*-type (16:1 to 24:1). The dsDNA binding activity of p53 DBD (30.0 pmol) in the absence of FA was taken as 0%. Data are shown as the means ± SEM for four independent experiments.

As *n*-octadecane had no effect on the activity, the free carboxyl moiety of FA is important for the inhibition of the dsDNA binding activity of p53 DBD. Among the *cis*-configuration of polyunsaturated C_{18} FA (1.20 nmol each) tested, oleic acid had the strongest inhibitory activity, the second strongest inhibitor was stearic acid, and the weakest was parinaric acid (18:4n-3) (Fig. 2B). These results suggested that PUFA, which have two or more double bonds, did not influence the activity.

To know the inhibitory effects of the hydrocarbon chain length of FA, C_{16} to C_{26}-saturated FA were investigated. As shown in Fig. 2C, the FA (1.20 nmol each) with more than 50% inhibitory activity were eicosanoic acid (20:0), heneicosanoic acid (21:0) and docosanoic acid (22:0), and the strongest inhibitor was docosanoic acid. Among C_{16} to C_{24}-monounsaturated FA (*cis*-type), 1.20 nmol of four FA such as *cis*-10-nonadecenoic acid (19:1n-9), *cis*-11-eicosenoic acid (gadoleic acid: 20:1n-9), *cis*-12-heneicosenoic acid (21:1n-9) and *cis*-13-docosenoic acid (erucic acid: 22:1n-9) inhibited the activity more than 50%, and *cis*-12-heneicosenoic acid had the strongest activity of p53 DBD inhibition (Fig. 2D). It was considered that there are suitable hydrocarbon lengths of FA for inhibition; in particular, the lengths of saturated and monounsaturated FA were C_{20} to C_{22} and C_{19} to C_{22}, respectively. Therefore, we concentrated on the properties of docosanoic acid and *cis*-12-heneicosenoic acid, which are the strongest saturated and monounsaturated FA inhibitors, respectively, in the latter part of this study.

To determine whether the FA resulted in binding to DNA or p53 DBD, the interaction of FA with dsDNA was investigated based on the thermal transition of dsDNA with or without FA. The melting temperature (Tm) of dsDNA with an excess amount of docosanoic acid or *cis*-12-heneicosenoic acid (100 μM) was measured using a spectrophotometer equipped with a thermoelectric cell holder. In the concentration range used, no thermal transition of Tm was observed, whereas ethidium bromide used as a positive control, a typical intercalating compound, produced a clear thermal transition. These results indicated that these compounds did not intercalate to dsDNA, and FA might directly bind to p53 DBD and inhibit its activity.

Effects of reaction conditions on long-chain FA inhibition. To determine the effects of a non-ionic detergent on the binding of docosanoic acid, *cis*-12-heneicosenoic acid and p53 DBD, Nonidet P-40 (NP-40) was added to the reaction mixture at various concentrations. The inhibitory effect of *cis*-12-heneicosenoic acid in the ranges of 0.60 nmol (i.e., 30.0 μM) on p53 DBD had no influence with the addition of NP-40 (Fig. 3); however, the p53 DBD inhibitory effect of docosanoic acid at 0.60 nmol was significantly reversed by the addition of NP-40 to the reaction mixture. The inhibitory reversion by NP-40 was concentration dependent, suggesting that docosanoic acid interacts with the hydrophobic region of p53 DBD protein. These results also suggested that the molecular binding mechanism of *cis*-12-heneicosenoic acid–p53 DBD binding was different from that of docosanoic acid–p53 DBD.

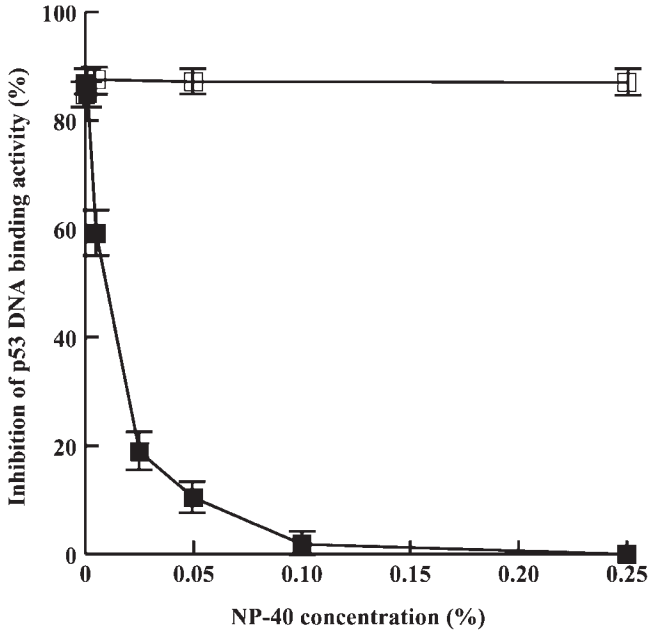

FIG. 3. Effect of detergents on the inhibition of human p53 DBD activities by FA. Nonidet P-40 (NP-40) was added to the reaction mixture at 0–0.25%. Docosanoic acid (22:0) (closed square) and *cis*-12-heneicosenoic acid (21:1n-9) (open square) were present at a concentration of 0.60 nmol. The dsDNA binding activity of p53 DBD (30.0 pmol) in the absence of FA was taken as 0%. Data are shown as the means ± SEM for three independent experiments.

We also tested whether an excess amount of a DNA analog, poly(rC) (2.00 pmol), or a protein, BSA (12.5 μM, 250 pmol), could prevent the inhibitory effects of FA. If FA bind to p53 DBD by non-specific adhesion, the addition of a nucleic acid and/or protein would be expected to reduce inhibitory activity. The fact that neither poly(rC) nor BSA influenced the inhibitory effects of FA suggests that the compound occurs selectively or binds to a specific site on p53 DBD and not to the nucleic acid.

Binding analysis of FA and human p53 DBD using surface plasmon resonance. To confirm the direct binding between FA and human p53 DBD, the kinetic parameters for docosanoic acid/*cis*-12-heneicosenoic acid and binding were determined using p53 DBD immobilized to the sensor chip in a BIAcore. Four different concentrations of docosanoic acid or *cis*-12-heneicosenoic acid (50 to 400 pmol, i.e., 1.25 to 10.0 μM) were used for the binding analysis. Because p53 DBD (565 pmol) was conjugated to the CM5 sensor chip, and then docosanoic acid or *cis*-12-heneicosenoic acid was added to the conjugated proteins, the response differences for the binding of docosanoic acid and *cis*-12-heneicosenoic acid to the protein were the same, approximately 200 RU (Fig. 4A and B). These FA dissociated very slowly from p53 DBD. The dissociation constants (K_D) of the binding of docosanoic acid and *cis*-12-heneicosenoic acid to the protein were determined to be 12.0 nM and 10.8 nM, respectively, from these data. The kinetic studies supported the suggestion that FA interact with p53 DBD directly.

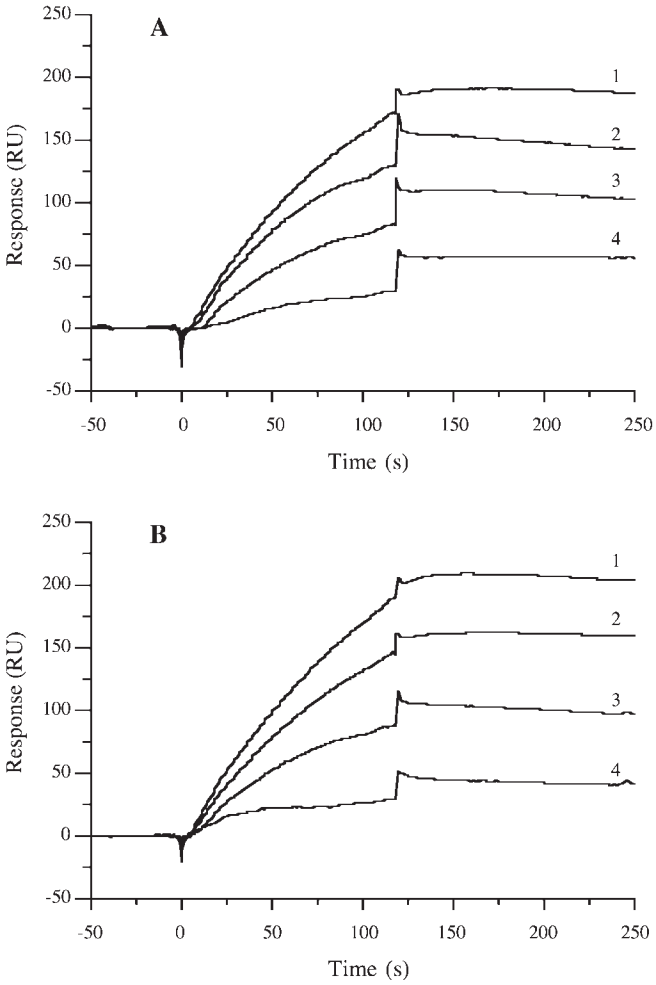

FIG. 4. BIAcore analysis of the binding of FA to immobilized human p53 DBD. p53 DBD (565 pmol) binding to docosanoic acid (22:0) (A) and *cis*-12-heneicosenoic acid (21:1n-9) (B) was detected by surface plasmon resonance signal (BIAcore, see Experimental procedures) and is indicated in response units. Four different concentrations of FA (curve 1, 10 μM (400 pmol); curve 2, 5 μM (200 pmol); curve 3, 2.5 μM (100 pmol); curve 4, 1.25 μM (50 pmol)) were injected over the protein for 120 S at 20 μl/min and dissociated for 130 S at 20 μl/min. The background resulting from the injection of running buffer alone was subtracted from the data before plotting.

Inhibitory effect of FA on the dsDNA binding activity of the mutant of human p53 DBD. Sequencing of the p53 gene in mammals, amphibians, birds, and fish has revealed five highly conserved domains, four of which fall within exons 5 through 8 (11): domain II (residues 117-142), domain III (residues 171-181), domain IV (residues 234-258), and domain V (residues 270-286). p53 is mutated in over half of all tumors. The overwhelming majority of these changes are found in the central DNA binding domain (i.e., p53 DBD) comprised of residues 94–312, which consists of domains II to V (5). Six tumorigenic mutations (R175, G245, R248, R249, R273 and R282) are most prevalent in the general population (5, 12), and these positions account for 22% of the 15,000 p53 mutations identified from human tumors and cell lines according to the database compiled by the Institut Curie (http://p53.free.fr/). In particular, R248 in domain IV is the most common mutation (13), therefore, an R248A mutant of human p53 DBD was prepared, and the inhibitory effect of FA was investigated as compared with the wild-type.

Figure 5 shows the inhibition dose-response curves of docosanoic acid and *cis*-12-heneicosenoic acid against the wild-type and R248A mutant of human p53 DBD (30.0 pmol).

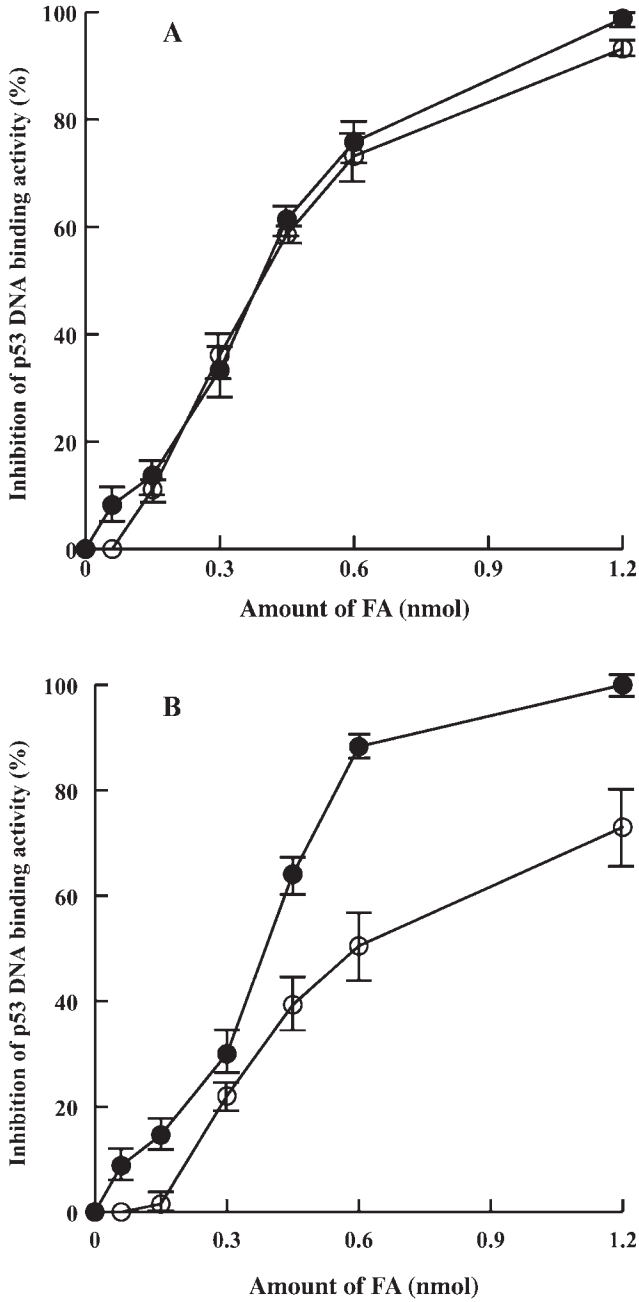

FIG. 5. Effect of FA on the inhibition of the R248A mutant of human p53 DBD activity. Inhibitory dose-response curves of docosanoic acid (22:0) (A) and *cis*-12-heneicosenoic acid (21:1n-9) (B) for the dsDNA binding activities of p53 DBD wild-type (30.0 pmol, closed circle) or R248A mutant (30.0 pmol, open circle). The dsDNA binding activity of p53 DBD (30.0 pmol) in the absence of FA was taken as 0%. Data are shown as the means ± SEM for three independent experiments.

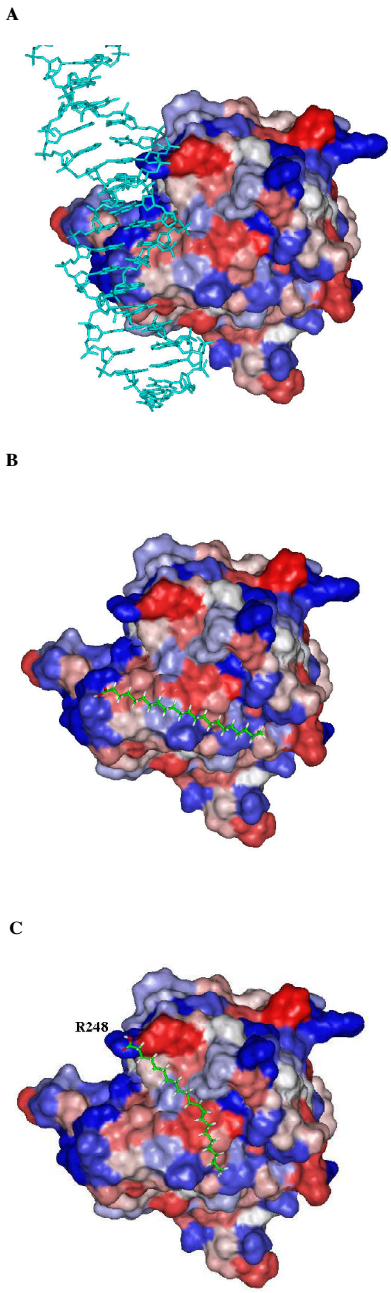

FIG. 6. Simulation of the long-chain FA interaction interface on human p53 DBD. (A) The complex of dsDNA and p53 DBD. (B) The complex of docosanoic acid (22:0) and p53 DBD. (C) The complex of *cis*-12-heneicosenoic acid (21:1n-9) and p53 DBD. Connolly surface and electrostatic potential of p53 DBD are shown, blue is a positive charge, and red is a negative charge. The carbons, oxygens and hydrogens of the structure of FA are indicated in green, red and white, respectively. The molecular docking of the DNA binding domain of human p53 DBD (PDB: 1TUP) was performed using the Affinity program within the Insight II software (Accelrys, San Diego, CA). The calculation used a CVFF force field in the Discovery program (Accelrys).

Both docosanoic acid (Fig. 5A) and *cis*-12-heneicosenoic acid (Fig. 5B) were effective at inhibiting the dsDNA binding activity of wild-type p53 DBD, with 50% inhibition observed at 0.41 and 0.36 nmol, respectively. The inhibitory effect of docosanoic acid on the activities of R248A mutant p53 DBD was almost as strong as that of the wild-type (Fig. 5A). On the other hand, inhibition of the activity of mutant p53 DBD by *cis*-12-heneicosenoic acid was 1.7-fold weaker than that of the wild-type (Fig. 5B). These results suggested that the p53-binding surface of docosanoic acid was different from that of *cis*-12-heneicosenoic acid; in particular, *cis*-12-heneicosenoic acid must bind to or interact with R248 in p53 DBD, but docosanoic acid might not influence R248.

Simulation of the docking of human p53 DBD with FA. As described above, the three-dimensional structure of human p53 DBD with or without FA such as docosanoic acid and *cis*-12-heneicosenoic acid was studied (Fig. 6). The crystal structure of the human p53 DBD in complex with consensus dsDNA is shown in Fig. 6A. A large immunoglobulin-like β-sandwich, forming a compact barrel-like structure, provides the basic scaffold for the conserved DNA binding surface (the blue area of p53 DBD in Fig. 6). This extended surface, rich in basic amino acids, consists of a loop-sheet-helix motif and two large loops (L2 and L3).

The docosanoic acid-binding site of p53 DBD was refined using Insight II / Discovery (Accelrys Inc.) (Fig. 6B). A cavity with a space (grid size, site of open size and site of cutoff size were 2.54 Å, 2.54 Å and 28.41 Å, respectively) where docosanoic acid, which is a linear carbon chain, can bind was searched for on the surface of the protein, and found in the hydrophobic area. Docosanoic acid was suggested to interact with the hydrophobic region of p53 DBD, because the complex of docosanoic acid and p53 DBD was reversed by a neutral detergent, NP-40 (Fig. 3). In these simulation results, the carboxyl end of docosanoic acid on p53 DBD was the same position as the minor groove of dsDNA on p53 DBD, because the free carboxyl group of FA was required for the inhibition of dsDNA binding activity (Fig. 2A).

The docking simulation of *cis*-12-heneicosenoic acid and p53 DBD is shown in Fig. 6C. As the inhibitory effect of *cis*-12-heneicosenoic acid on the R248A mutant of p53 DBD was weaker than that on the wild-type (Fig. 5), the carboxyl group of *cis*-12-heneicosenoic acid could interact with the residue of R248, and the V-like-shaped alkyl chain region interacted with the hydrophobic surface of p53. It was suggested that *cis*-12-heneicosenoic acid bound to the residue of R248 of p53 DBD and inhibited dsDNA-binding activity by competing with dsDNA. Since the molecular shape of *cis*-12-heneicosenoic acid (grid size, site of open size and site of cutoff size were 2.54 Å, 6.25 Å and 25.35 Å, respectively) is different from docosanoic acid, it might be considered that there are two FA-binding pockets in p53 DBD. The size of the pockets may be a key to explain these inhibition characteristics by FA. This is the first report to find that C_{20} to C_{22} long-chain FA could bind to human p53 directly, and inhibit or regulate the dsDNA binding activity of p53.

Free FA play important physiological roles in many tissues. For example, the impairment of insulin-mediated glucose uptake and glycogen synthesis in muscle and the potentiation of

glucose-stimulated insulin secretion in pancrearic islets have been considered as a result of the intracellular metabolism of free FA to long-chain acyl-CoA esters. However, it is also suggested that free FA may act as ligands for cell-surface receptors because FA derivatives, such as prostaglandins and leukotrienes, have specific cell-surface G protein-coupled receptors. PUFA has been shown to promote apoptosis in pancreatic cancer cells (14), and in fibroblast and lymphoblastoid cell lines (15). However, our results suggested that saturated and monounsaturated FA might act as regulators or inhibitors of transcription by p53. It was considered that apoptosis induced by FA might be independent of the p53 pathway.

The p53 tumor suppressor protein responds to a variety of cellular stresses, including DNA damage, UV irradiation, hypoxia and aberrantly activated oncogenes, and may induce cell-cycle arrest or apoptosis (16–18). However, the molecular mechanisms of the various biological activities of p53 have not been characterized. In this study, we found that saturated and monounsaturated (*cis*-type) long-chain FA such as docosanoic acid and *cis*-12-heneicosenoic acid strongly inhibited the dsDNA binding activity of human p53 DBD. Since these FA are present in the inner membrane of human cells (19), FA might regulate the biological activities of p53 to achieve complexes of FA and p53 *in vivo*. The *in vivo* regulation mechanism of p53 by FA will be a future study.

ACKNOWLEDGMENTS

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Low-Density Lipoproteins Are More Electronegatively Charged in Type 1 Than in Type 2 Diabetes Mellitus

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ABSTRACT: Multifactorial etiology is involved in premature atherosclerosis related to diabetes. Most of the mechanisms that are responsible for the etiology in diabetes have remained unsolved so far. Type 1 diabetes is associated with a favorable lipid pattern and with microangiopathy, which is not true for type 2 diabetes, which is related to dyslipidemia and macroangiopathy. The aim of this work was to evaluate the degree of LDL modification related to the types of diabetes. The question is whether the LDL could be differently modified since the pathogenesis of type 1 and type 2 diabetes is different. Thirty-one type 1 (19 male and 12 female) and thirty type 2 (18 male and 12 female) diabetic patients were included in this study. Isolated LDL was analyzed by capillary electrophoresis for diene conjugate content and for electronegativity. LDL from type 1 diabetes subjects showed the highest electrophoretic mobility (P = 0.000). Instead, the diene conjugates contents were higher in the type 2 patients with HbA1c levels >8 % (P = 0.007). In conclusion, the increased diene content in type 2 diabetic subjects in poor glycemic control and the highest LDL mobility found in type 1 subjects show that the LDL undergoes different modifications. In type 2 patients, electronegative LDL are in a state of higher susceptibility to oxidation, whereas in type 1 subjects the finding of electronegative lipoproteins could provide an index of the relative atherogenicity of circulating LDL, especially as LDL has higher electrophoretic mobility than normal subjects.

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Multifactorial etiology is involved in premature atherosclerosis related to diabetes. Most of the mechanisms that are responsible for the etiology in diabetes have remained unsolved so far. Without any doubt, a major factor is an impaired lipoprotein metabolism. Hypertriglyceridemia, increased postprandial lipemia, and the presence of small dense LDL and decreased HDL levels (1–4) are the common abnormalities that often occur in diabetes. Focus has therefore turned to qualitative changes in lipoprotein metabolism rather than simply lipoprotein concentrations. Modified or aberrant forms of lipoproteins, including glycated lipoproteins, favor the progression of atherosclerosis (5). LDL is the lipoprotein component responsible for transferring either exogenous or endogenous lipids to peripheral tissues. Oxidation of the LDL particles enhances its atherogenicity (6), and the interaction of oxidation and the excess glycosylation seen in diabetes (7) may be the source of the cardiovascular risk (8). The oxidative modification of LDL can exert cytotoxic and immunogenic effects (9). Additionally, oxidized LDL contributes to atherogenesis by its ability to trigger an immune response leading to the formation of immune complexes (10). The electrophoretic mobility linked to the measure of the amount of baseline diene conjugation (BDC) in LDL is one of the more reliable indicators of LDL modification (11). The measure of BDC in LDL is a clinically applicable method to estimate *in vivo* LDL oxidation (12). Strong correlation between the titer of autoantibodies against oxidized LDL and results of HPLC and NMR studies indicated that BDC-LDL was a specific measure of oxidized LDL *in vivo* (13).

Oxidation, like glycosylation, is a process that increases the negative charge of LDL (14). Modifications that increase the net negative charge may have important metabolic consequences (15,16). The advanced glycation end-products (AGE) process is one of the main pathogenic mechanisms linked to the development of diabetic complications (17), and both oxidized LDL and AGE-LDL have been detected (18) in diabetes.

This study was undertaken with the aim of evaluating the degree of LDL modification related to the types of diabetes. Type 1 diabetes has a different pathogenesis from type 2 diabetes, and although there are similarities between the diseases such as hyperglycemia, inferences cannot be made from one type to the other for all risk factors, such as lipids and obesity (19). Type 1 diabetes is associated with a favorable lipid pattern and with microangiopathy; this is not true for type 2 diabetes, which is related to dyslipidemia and macroangiopathy (20). The question is whether the LDL could be differently modified because the pathogenesis of type 1 and type 2 diabetes is different. To this purpose, we have measured the electronegativity and diene conjugates content in LDL isolated in two groups of diabetic subjects.

EXPERIMENTAL PROCEDURES

Study subjects. Thirty-one type 1 (19 male and 12 female) and thirty type 2 (18 male and 12 female) diabetic patients were included in this study. They were recruited from the diabetes clinic at the San Giovanni Battista Hospital in Turin (Italy). They were enrolled based on good (glycated hemoglobin [HbA_{1c}] < 8%) and poor (HbA_{1c} > 8%) glycemic control. Di-

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Abbreviations: AGE, advanced glycation end-products; BDC, baseline diene conjugation; BMI, body mass index; CE, capillary electrophoresis; EOF, electroendosmotic flow; glyc-LDL, glycosylated LDL.

abetes was defined according to the National Diabetes Data Group criteria (21). For the type 1 diabetic subjects mean age was 42 ± 15.7 yr, body mass index (BMI) was 25 ± 3.9 kg/m^2, and known diabetes duration was 20 ± 10.5 yr. For the type 2 diabetic subjects control mean age was 61 ± 10.5 yr, BMI was 28 ± 5.3 kg/m^2, and known diabetes duration was 9.2 ± 7.3 yr. Antropometric characteristics, glycemic control parameters, and lipid profiles of type 2 diabetic patients are shown in Table 1.

All the type 2 diabetic patients were on hypoglycemic oral agents, and insulin therapy was administered to type 1 diabetic patients. None of the patients were taking any other drugs or vitamins, or had any disease known to influence lipoprotein metabolism. To exclude an influence of the diet on LDL mobility, all the subjects were given a common standard diet 3 mon before blood drawing.

The type 1 diabetic subjects with a value of Lp(a) exceeding 15 mg/dL were excluded.

Informed consent has been obtained from all the enrolled subjects and the reported investigations have been carried out in accordance with the principle of the Declaration of Helsinki (22).

Blood samples were collected from all participants in the fasting period.

Twenty-six healthy subjects matched for gender (17 males and 9 females) and with a normal lipid profile were enrolled as control group.

Biochemical analyses. Glucose was determined by a standardized automatized enzymatic method, glucose oxidase (Sentinel CH, Milan, Italy), adapted to a Hitachi 911 autoanalyzer. Cholesterol and triglyceride concentrations were determined from plasma and lipoprotein fraction by enzymatic methods (Sentinel CH) adapted to a Hitachi 911 autoanalyzer.

Isolation of LDL. Venous blood was collected from all participants into EDTA-containing Vacutainer tubes, and plasma was separated by low-speed centrifugation for 15 min at 4°C. LDL was isolated by preparative sequential ultracentrifugation (23) in a Beckman L8-70M ultracentrifuge (Beckman Instruments Inc., Fullerton, CA). Plasma was adjusted to a density of 1.019 kg/L with KBr solution and ultracentrifuged at 121,000 g for 20 h at 18°C in a Beckman 70.1 rotor. After centrifugation, the VLDL fraction was removed by the tube slicing and the infranate was adjusted to a density of 1.063 kg/L and ultracentrifuged at 148,000 g for 21 h at 18°C. To avoid oxidative modification of lipoproteins, 1 mmol/L EDTA was added to all KBr solutions in the two sequential ultracentrifugations. The fraction containing LDL was removed by tube slicing and dialyzed through Sephadex G-25 columns (Pharmacia, Uppsala, Sweden) against buffers used in the subsequent experiment.

In order to rule out the presence of Lp(a) in the samples containing LDL, we performed a Lp(a) determination in all the fractions with an immuno-nephelometric method (Behring, Italy).

Chromatographic assays. HbA_{1c} was routinely determined by standardized affinity HPLC (Bio Rad, Milan, Italy). The reference range in our laboratory is 4.0–5.8%.

Capillary electrophoresis. Capillary electrophoresis (CE) was performed with a Beckman P/ACE 5510 system fitted with a diode array detector. An untreated fused silica capillary tube with a 75 µm i.d. was used. The total length of the capillary tube was 57 cm (50 cm to the detector). The capillary was operated at 20°C. An applied voltage of 350 V/cm was used in all CE separations. CE was performed as described by Stocks *et al.* (11). The cathode and anode electrolytes and the capillary run buffer were 40 mM methylglucamine-Tricine, pH 9.0. LDL samples were injected by low pressure for 4 s. Dimethylformamide was injected as an electroendosmotic flow (EOF) marker for 1 s. A voltage of 24 kV was applied ramping over 0.8 min. Migration of LDL particle was monitored at 200 and 234 nm. The amount of conjugated dienes is obtained from the percentage of the height of LDL peak at 234 nm related to the height of LDL peak at 200 nm.

Calculation of electrophoretic mobility. The electrophoretic mobility of LDL (µ) was calculated using the following equation (24): $\mu = L_c L_d / U(1/t_{sample} - 1/t_{EOF})$, where L_c is the total length of the capillary (cm), L_d is the length of the capillary from injector to detector (cm), U is the applied voltage (V), t_{sample} is the migration time of LDL (s), and t_{EOF} is the migration time of the EOF marker (s). The higher the migration rate of LDL is, the more negative is the result. The negative result mirrors the net charge of the molecule, which is, of course, negative.

Statistical analysis. Data were expressed as means ± SD unless otherwise indicated. Student's *t*-test was used to compare groups when variables were normally distributed. Pearson's correlation coefficients were used to describe relationships between variables. In all cases, *P* less than 0.05 was considered statistically significant. Triglyceride values were log-transformed before analyses.

TABLE 1
Anthropometric Characteristics, Glycemic Control Parameters, and Lipid Profiles of Type 1 and 2 Diabetic Patients

	Type 1 diabetic subjects	Type 2 diabetic subjects	*P*
Sex (M/F)	31 (19/12)	30 (18/12)	
Age (yr)	42 ± 15.6	61 ± 10.5	0.000
Diabetes duration (yr)	19.8 ± 10.5	9.2 ± 7.3	0.000
BMI (kg/m^2)	25 ± 3.9	28.2 ± 5.3	0.009
HbA1c (%)	7.70 ± 1.55	8.04 ± 2.04	0.470
Glucose (mg/dL)	185 ± 76	167 ± 64	0.317
Total cholesterol (mg/dL)	179 ± 33.5	200 ± 37.6	0.0399
Log [triglycerides (mg/dL)]	1.92 ± 0.19	2.13 ± 0.29	0.04
LDL cholesterol (mg/dL)	101 ± 26	124 ± 34	0.011
HDL cholesterol (mg/dL)	59 ± 15	50 ± 20	0.086

RESULTS

Anthropometric characteristics, glycemic control parameters, and lipid profiles of type 1 and 2 diabetic subjects are shown in Table 1. Type 1 and 2 diabetic patients had $HbA1_c$ levels of 7.70 ± 1.56% and 8.04 ± 2.04%, respectively. Type 2 dia-

betic patients had a higher BMI value and shorter diabetes duration than type 1 diabetic patients.

The LDL electrophoretic mobility mean for the type 1 diabetic group was higher than that for the type 2 diabetic group ($-1.2669 \pm 0.056 \times 10^{-4}$ vs. $-1.0905 \pm 0.0724 \times 10^{-4}$ cm^2 V^{-1} s^{-1}, respectively, $P = 0.000$; Table 2). In both diabetic groups, LDL electrophoretic mobility and the content of conjugated dienes were significantly higher than that found in the control group ($P < 0.001$, data not shown). No significant difference in diene conjugated was observed in the LDL isolated from type 1 and 2 diabetic patients ($P = 0.293$).

The diabetic subjects were further divided according to their $HbA1_c$ levels in two subgroups. The $HbA1_c$ cut-off value was set at 8.0%. In diabetic subjects with $HbA1_c$ < 8.0%, only the electrophoretic mobility of isolated LDL in the type 1 diabetic subjects was higher than that observed in the type 2 subjects ($-1.272 \pm 0.06 \times 10^{-4}$ vs. $-1.077 \pm 0.053 \times 10^{-4}$ cm^2 V^{-1} s^{-1}, respectively, $P = 0.000$). The diene conjugates contents were similar (6.76 ± 1.01% for type 1 diabetes vs. 6.69 ± 0.93% for type 2 diabetes, $P = 0.833$; Table 3).

Similarly, when LDL isolated from type 1 and 2 diabetic subjects with $HbA1_c$ > 8.0% was compared, the electrophoretic mobility was increased in the type 1 diabetic group (-1.259 ± 0.05 ? 10^{-4} vs. $-1.1034 \pm 0.088 \times 10^{-4}$ cm^2 V^{-1} s^{-1}). On the contrary, the diene conjugates contents were higher in the type 2 patients (5.81 ± 0.39% for type 1 diabetes vs. 6.60 ± 0.87% for type 2 diabetes). In both cases the statistical difference was reached ($P = 0.000$ for the electrophoretic mobility, and $P = 0.008$ for diene conjugates; Table 4).

After splitting each diabetic group into two subgroups on the basis of the hemoglobin levels, it was observed that among type 1 patients the diene conjugates of LDL were higher in the subjects who had $HbA1_c$ < 8.0% (6.76 ± 1.01% vs. 5.81 ± 0.39%, $P = 0.0045$).

The electrophoretic mobility and the diene content of LDL isolated from subjects with type 2 diabetes mellitus were not statistically different.

DISCUSSION

Our study was designed to highlight the electrophoretic characteristics of LDL isolated from subjects affected by diabetes and to determine whether the susceptibility to oxidation of the lipoprotein particle could be modified by a glucose-rich environment and other factors related to diabetes. We expected to find some differences in the quality of LDL according to the type of diabetes, because the pathogenesis and the duration (Table 1) of the two diseases are different.

Our work highlights two important phenomena: an increased electrophoretic mobility of LDL in type 1 diabetic patients relative to type 2 diabetic subjects, and an increased diene conjugates content in LDL of type 2 diabetic subjects in poor glycemic control relative to those affected with type 1 diabetes. Furthermore, the diabetic disease modifies the circulating LDL, in accordance to what was found in the control group, whose LDL had electrophoretic mobility and conjugated dienes far lower than in diabetic subjects. The electrophoretic mobility is one of the more reliable indicators of LDL modification. The more electronegative mobility found in LDL isolated from type 1 diabetic subjects agrees with our previous work (25), in which they were related to the increase of the so-called LDL(-) (26), which is also detectable in normal subjects, although in small amounts (27). One can argue that diene conjugates is the major determinant of electrophoretic mobility in type 1 diabetes. Instead, diene conjugates in type 1 diabetes are similar to those in type 2 diabetes. In other words, factors other than glucose having capability in modifying lipoproteins should be implicated.

In type 1 diabetes the diene conjugates decreased with the impairment of the glucose metabolism. That is, at high values

TABLE 2
Electrophoretic Mobility and Diene Conjugates of LDL in Type 1 and 2 Diabetes Mellitus Patients and in Healthy Subjects

	Type 1 diabetic subjects ($n = 31$)	Type 2 diabetic subjects ($n = 30$)	P^a	Control group subjects ($n = 26$)
Electrophoretic mobility ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})	-1.2669 ± 0.056^b	-1.0905 ± 0.072^b	0.000	–0.99421 ± 0.083
Diene conjugates (%)	6.39 ± 0.94^b	6.64 ± 0.88^b	0.293	5.39 ± 0.44

[a]Type 1 vs. type 2 diabetic subjects.
[b]$P < 0.001$ vs. control group.

TABLE 3
Electrophoretic Mobility and Diene Conjugates of LDL in Type 1 and 2 Diabetes Mellitus with Hb1c < 8%

	Type 1 diabetic subjects ($n = 19$)	Type 2 diabetic subjects ($n = 15$)	P
Electrophoretic mobility ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})	–1.272 ± 0.061	–1.077 ± 0.053	0.000
Diene conjugates (%)	6.76 ± 1.01	6.69 ± 0.93	0.833

TABLE 4
Electrophoretic Mobility and Diene Conjugates of LDL in Type 1 and 2 Diabetes Mellitus with Hb1c > 8%

	Type 1 diabetic subjects ($n = 12$)	Type 2 diabetic subjects ($n = 15$)	P
Electrophoretic mobility ($\times 10^{-4}$ cm^2 V^{-1} s^{-1})	–1.258 ± 0.049	–1.103 ± 0.087	0.000
Diene conjugates (%)	5.81 ± 0.39	6.60 ± 0.87	0.007

of HbA1c we observed a trend toward low levels of dienes (from 5.81 ± 0.39% to 6.77 ± 1.01%, $P = 0.0045$ for type 1 diabetes). In agreement with that, the negative correlation found between HbA1c and diene conjugates explains that glycosylation can offer lipoproteins more resistance to the further oxidation process as other authors have suggested (28).

At a value of HbA1c > 8% type 2 diabetic patients had a significantly higher diene conjugates level than type 1 subjects. The diene conjugates play a major role in type 2 diabetic patients, who often have dyslipidemia, which provides excellent substrates for lipid peroxidation. Therefore, the increased diene contents in type 2 diabetes LDL isolated from subjects in bad glucose control could be due to the metabolic alterations, which are typical of type 2 diabetes. The measure of conjugated dienes in circulating LDL is an indicator of oxidized LDL *in vivo* (12), which in type 2 subjects are related to the demonstrated higher prevalence of subjects of a more atherogenic LDL phenotype (29), because small dense LDL particles are more susceptible to oxidation (30). Oxidizability of plasma lipoproteins in diabetic patients not due to glycation (31) was investigated by M. Kalousová *et al.* (32), who found that both AGE and advanced oxidation protein products are elevated in patients with diabetes mellitus, with marked differences between patients with diabetes mellitus type 1 and type 2. The increase of both parameters is more pronounced in patients with type 2 diabetes mellitus. This might indicate the presence of oxidative stress in both types of diabetes mellitus with its increase in type 2. Oxidative stress probably also plays a more important role in AGE formation in diabetes mellitus type 2 than in type 1, where AGE formation depends more on the compensation of the disease.

In conclusion, the finding of more electronegative LDL assumes different meanings according to the type of diabetes. In type 2 patients electronegative LDL are in a state of higher susceptibility to oxidation, whereas in type 1 subjects the finding of electronegative lipoproteins could provide an index of the relative atherogenicity of circulating LDL, especially as LDL has higher electrophoretic mobility than normal subjects. The increased negative charge of type 1 diabetic LDL could be related to underlying atherosclerosis-related complications developed during long-duration diabetes or to compositional abnormalities. Structural endothelial proteins become modified by AGE and trap circulating lipoproteins (33). Modifications of lipoproteins enhance their atherosclerotic behavior.

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Dietary Fatty Acids Early in Life Affect Lipid Metabolism and Adiposity in Young Rats

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ABSTRACT: The purpose of this study was to evaluate the effects of four isoenergetic diets of differing fat composition on blood lipid profile and adiposity in young rats. Diets containing different lipid sources—partially hydrogenated vegetable oil (PHVO), palm oil (PO), canola oil (CO), and soy oil (SO)—were fed to lactating rats during the 21 days of lactation, and then fed to young males following weaning until the 45th day of life. *In vivo* lipogenesis rate (LR), lipid content (LC), relative level of FA, and the activity of lipoprotein lipase (LPL) enzyme were measured in epididymal adipose tissue (EPI). Fasting blood lipoproteins and LC in the carcass were also appraised. Body weight of PO and PHVO groups was significantly higher than CO and SO groups from day 14 of lactation to day 45, despite the lower food intake in the PHVO group. PO and PHVO groups presented higher LR and LC in EPI than SO and CO groups. Carcass fat content was significantly higher in PHVO and PO groups than in CO and SO groups. The LPL activity in EPI was unaffected by dietary lipids. PHVO group had increased total cholesterol and TAG concentrations in comparison with the PO group, and significantly lower HDL level compared with the other groups. These results show that the kind of FA in the dietary lipid offered early in life can affect lipid metabolism and adiposity.

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In past years, considerable interest has focused on dietary fat, an essential nutrient that can increase the risk of certain chronic diseases if consumed in excess (1). Several studies have been conducted in a search for possible effects of the level of dietary fat, fat type (animal vs. vegetable), and FA classes (e.g., saturated, monounsaturated *cis* and *trans*, and polyunsaturated) on various chronic diseases (2,3,4). More recently, interest has focused on the FA composition of dietary fats early in life with a view toward preventing a further increase of childhood obesity in future generations (5).

White adipose tissue is the most important extrahepatic site regulating *in vivo* lipid metabolism. TAG deposition in this tissue is related to the *in situ de novo* synthesis, the uptake of FA from lipoproteins derived from dietary lipids and liver, and the lipolysis rate (6). Some studies have demonstrated that the dietary FA type can influence the metabolism of adipose tissue (6,7). Doucet *et al.* (8) observed that diets rich in saturated FA (SFA) and monounsaturated FA (MUFA) lead to a higher body adiposity than those rich in PUFA. Only one study, performed on rats during the gestation/lactation period, and subsequently on post-weaning pups eating the same diet their mother had consumed, has emphasized the sensitivity of adipose tissue to the FA composition of ingested fats (9).

The question of how the lipid composition of the diet can affect body adiposity early in life is still controversial, particularly in studies with normolipidic diets containing different compositions of FA.

Taking into consideration that there are only a few studies on the metabolic effects of the FA during the period of growth and development, particularly involving *trans* FA (tFA), whose dietary consumption has been increasing in recent years, we aimed to investigate the possible metabolic effects of different sources of dietary fats. We compared omega-6 PUFA, MUFA, SFA, and tFA on lipid levels and adipose tissue metabolism of young rats.

MATERIALS AND METHODS

Animals, diets, and general procedures. Pregnant female Wistar rats weighing 180–220 g, obtained from the animal breeding unit of the Institute of Nutrition of the Federal University of Rio de Janeiro, were maintained under controlled conditions (24 ± 1°C and 12 h light/12 h dark cycle). On the parturition day (day 0 of lactation), litters were weighed and adjusted to six male pups per dam. The lactating dams and their respective litters were divided into four groups according to the experimental diets, which were isocaloric (all diets provided 4.1 kcal/g of dry diet) but differed in the FA profile. After weaning (day 21 of life), the pups received the same diets as their mothers until the 45th day of life. The soy oil (SO) group received a diet containing 7% soy oil; the partially hydrogenated vegetable oil (PHVO) group received 6% partially hydrogenated vegetable oil plus 1% soy oil; the palm oil (PO) group received 5% palm oil plus 2% soy oil, and the canola oil (CO) group received 6% canola oil plus 1% soy oil. Soy oil was included in each diet to adjust the diet to the requirement for essential FA. The composition of each diet com-

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Abbreviations: AA, arachidonic acid; CO, canola oil; EPI, epididymal adipose tissue; LPL, lipoprotein lipase; LR, lipogenesis rate; MUFA, monounsaturated FA; PHVO, partially hydrogenated vegetable oil; PO, palm oil; PUFA, polyunsaturated FA; SFA, saturated FA; SO, soy oil; TC, total cholesterol; tFA, trans FA.

TABLE 1
Composition of Diets[a]

Constituents (g/kg)	SO	CO	PO	PHVO
Casein (vitamin-free)	150.0	150.0	150.0	150.0
Cornstarch	679.49	679.49	679.49	679.49
Soy oil[a]	70.0	10.0	20.0	10.0
Hydrogenated fat[a]	—	—	—	60.0
Palm oil	—	—	50.0	—
Canola oil[b]	—	60.0	—	—
Butylhydroquinone-BHT	0.014	0.014	0.014	0.014
Cellulose	50.0	50.0	50.0	50.0
Vitamin mix[c]	10.0	10.0	10.0	10.0
Mineral mix[d]	35.0	35.0	35.0	35.0
Choline bitartrate	2.5	2.5	2.5	2.5
L-Cystine	1.8	1.8	1.8	1.8

[a]Soy oil and hydrogenated fat were obtained from Lisa/Ind. Brazil.
[b]Hydrogenated vegetable oil was obtained from Gessy Lever, São Paulo, Brazil.
[c]Vitamin mix contents (g/kg diet): thiamin HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; niacin, 3.0; calcium pantothenate, 1.60; folic acid, 0.20; biotin, 0.02; vitamin B12, 2.5; vitamin A palmitate (500.0 IU/g), 0.80; vitamin E acetate (500 IU/g), 15.0; vitamin D3 (400.000 IU/g), 0.25; vitamin K1, 0.75. AIN-93 vitamin mix, DYETS 310025, obtained from Dyets Inc., Bethlehem, PA.
[d]Mineral mix provided (g/kg): calcium, 357.0; phosphorus, 250.0; potassium, 74.6; sodium, 74.0; sulfur, 300; magnesium, 24.0; iron, 5.21; copper, 0.3; manganese, 0.63; zinc, 1.65; chromium, 0.27; iodine, 0.01; selenium, 0.01; boron, 0.08; molybdenum, 0.01; silicon, 1.45; nickel, 0.03; lithium, 0.02; vanadium, 0.007. AIN-93 mineral mix, DYETS 210025, obtained from Dyets Inc.. SO, 7.0% soy oil (AIN-93); PHVO, 6.0 % partially hydrogenated vegetable oil plus 1% soy oil (AIN-93); CO, 6% canola oil plus 1% soy oil (AIN-93); PO, 5.0% palm oil plus 2% soy oil (AIN-93).

plied with the American Institute of Nutrition (10) recommendation and is shown in Table 1. The FA composition of the diets is summarized in Table 2. Diets were prepared as pellets and stored at 4°C until use. Rats had free access to diet and water throughout the experimental period. Food consumption was measured daily and body weight weekly from day 1 to 45 of life. The young male rats were killed on the 45th day of life according to two different procedures: (a) six rats in each group were killed after an overnight fast, between 10:00 and 12:00 a.m., by guillotine. Immediately after decapitation, the blood was collected in tubes containing EDTA (1 mmol/L) and used for plasma metabolite measurements. Samples of epididymal white adipose tissue (EPI) were quickly extracted and stored at –70°C for determination of lipoprotein lipase (LPL) activity. The carcasses were stored at –70ºC for later lipid analysis; (b) the other six rats were decapitated 1 h after intraperitoneal administration of 3 mCi of ^3H_2O, and the EPI was removed and stored at –70ºC for later measurement of LR and LC. All experimental protocols and procedures were approved by the University's Experimental Research Committee.

TABLE 2
FA Composition (%) in Experimental Diets

FA	SO	CO	PO	PHVO
Total SFA	15.13	11.67	43.25	22.46
14:0	0.06	0.98	0.84	0.07
16:0	11.58	8.06	39,00	10.24
18:0	3.49	2.62	3.41	12.15
trans MUFA	—	—	—	14.12
18:1 *trans* n-9/n-7 isomers	—	—	—	14.12
Total *cis* MUFA	22.60	57.46	33.5	25.08
18:1n-9	21.90	57.07	33.0	19.92
18:1 (other *cis*-isomers[a])	—	—	0.41	4.82
20:1	0.44	0.39	0.09	0.32
24:1	0.26	—	—	0.02
trans PUFA	—	—	—	0.69
18:2n-6 *tc*	—	—	—	0.17
18:2n-6 *ct*	—	—	—	0.40
18:2n-6 *tt*	—	—	—	0.12
Total PUFA	59.18	28.39	22.89	23.36
18:2n-6	53.15	23.46	21.18	20.75
18:3n-3	6.03	4.92	1.71	2.66
Total trans	—	—	—	14.81

[a]Includes all positional *cis*-isomers of 18:1 except 18:1n-9. For abbreviations, see Table 1.

FA analysis. Lipid extraction, saponification, and methylation of FA in the experimental diets and EPI were performed in duplicate using 0.5-g samples according to the method of Lepage and Roy (11), which includes treatment with 2 mL of 4:1 (vol/vol) methanol:benzene solution and addition of 200 µL of acetyl chloride under light agitation. FAME were quantified with GLC in a Perkin Elmer autosystem XL chromatograph with an ionizable flame detector and Turbochrom software (PerkinElmer, Norwalk, CT). FA were separated on a 100-m × 0.25-mm × 0.20-µm capillary column (SP2560, Supelco, Bellefonte, PA). Hydrogen was used as carrier gas. Injection and detection temperatures were 260°C and 280°C, respectively. The run temperature was programmed to start at 135°C for 5 min, with a 2°C increase per minute up to 195°C. Thereafter, the temperature increased 4°C per minute up to 240°C and was kept at this level for 2.5 min. Total run time was 45 min. The carrier gas pressure was 32 psi. The split ratio was 1:70. Esters were identified by comparing their retention times with those of standard FA. Results are expressed as percentage of total FA.

Measurements of LPL activity and protein content. LPL activity in epididymal adipose tissue was measured using the method of Nilsson-Ehle and Chotz (12) as modified by Llobera *et al.* (13). Tissue protein content was determined as described by Lowry *et al.* (14).

Measurements of in vivo lipogenesis and fat content. For measuring the *in vivo* lipogenesis rate, ^3H_2O (3 mCi) was injected intraperitoneally and the rats were killed by decapitation 1 h later. The EPI was removed and weighed, and 1-g aliquots were saponified in 3 mL 30% KOH. Lipids were extracted in petroleum ether for lipid extraction (15) and the radioactivity in the lipid fraction was measured. The LR was estimated as described by Robinson and Williamson (16). Carcasses were shaved, softened, and homogenized after removal of the gastrointestinal tract (17) to determine the carcass lipid content. Lipid was extracted from 3-g aliquots with petroleum ether and determined gravimetrically (15). Lipid content was determined as the difference between the weight of the vial containing the dry sample and the weight of the empty dry vial.

Plasma metabolites. Blood samples were taken by decapitating rats after an overnight fast of 12 h. Blood was collected in tubes containing EDTA (1 mmol/L), and plasma was separated immediately by centrifugation. Plasma total cholesterol (TC), HDL cholesterol, and TAG concentrations were determined by enzymatic methods with commercial kits (Gold Analisa Diagnóstica Ltda, Belo Horizonte, MG, Brazil).

Statistical analysis. The results were expressed as mean ± SEM, and statistical comparison among groups was done by a one-way ANOVA. Differences between means were tested for significance by Duncan's multiple range test ($P \leq 0.05$).

RESULTS

No significant differences were observed in maternal food intake and body weight of the experimental groups during lactation (data not shown). Evaluation of the post-weaning food intake (in g/100 g body weight) revealed that, immediately after weaning, the PO group consumed a significantly greater amount of food, followed by SO and CO groups. From the second post-weaning day onward, food intake was significantly lower in the PHVO group than in the other groups. However, from day 42 on, no significant differences were found among the dietary groups (Fig. 1). Regarding body weight, pups of PO and PHVO groups showed a significantly higher body weight than the others from day 14 on, and these differences persisted after weaning until the end of the experimental period (day 45) (Fig. 2). In contrast, no significant difference was observed between the SO and the CO groups. The carcass fat content (g/100 g) was significantly higher in the PO (8.97 ± 0.32) and PHVO (9.43 ± 0.88) groups compared with CO (3.67 ± 0.10) and SO (4.91 ± 0.48) groups.

The FA composition of EPI is summarized in Table 3. It is readily apparent that the FA composition of the EPI varied with

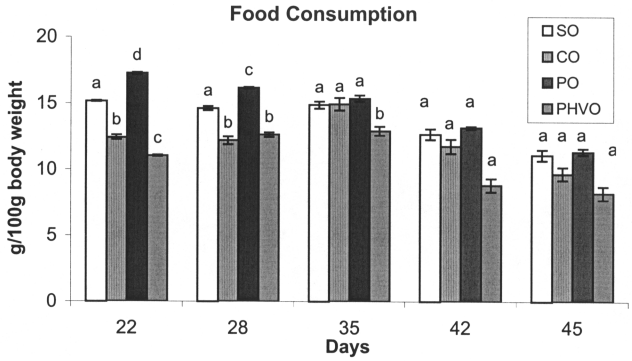

FIG. 1. Effects of dietary fat on food consumption in rats fed SO, CO, PHVO, or PO diets for 45 d. Values are mean ± SEM of six rats per group. Different superscript letters indicate significant differences from one another on the same day at $P \leq 0.05$ as determined by Duncan's test. SO, 7% soy oil; CO, 6% canola oil plus 1% soy oil; PHVO, 6% partially hydrogenated vegetable oil plus 1% soy oil; PO, 5% palm oil plus 2% soy oil.

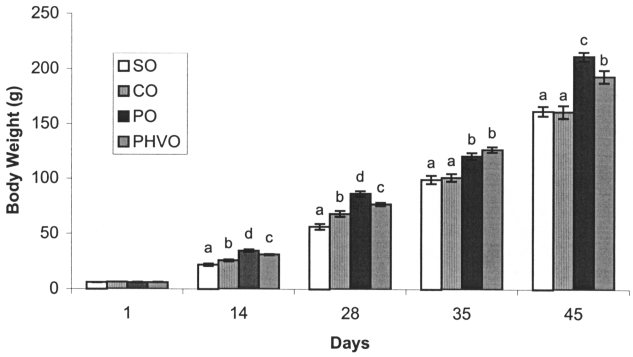

FIG. 2. Body weight (g) of the male pups during lactation (1 and 14 d) and after weaning (21–45 d). Values are mean ± SEM of six rats per group. Different superscript letters indicate significant differences from one another on the same day at $P \leq 0.05$ as determined by Duncan's test. SO, 7% soy oil; CO, 6% canola oil plus 1% soy oil; PHVO, 6% partially hydrogenated vegetable oil plus 1% soy oil; PO, 5% palm oil plus 2% soy oil.

TABLE 3
FA Composition (%) of Total Lipids in Epididymal Adipose Tissue[a]

FA	SO	CO	PO	PHVO
Total SFA	31.62 ± 0.42[a]	30.87 ± 0.31[a]	36.79 ± 0.28[b]	32.20 ± 0.25[a]
14:0	2.08 ± 0.05	2.09 ± 0.03	2.03 ± 0.04	1.96 ± 0.07
15:0	0.05 ± 0.01[a]	0.11 ± 0.01[ab]	0.07 ± 0.02[a]	0.13 ± 0.01[b]
16:0	26.16 ± 0.29[a]	26.37 ± 0.34[a]	31.96 ± 0.47[b]	26.52 ± 0.55[a]
18:0	3.33 ± 0.07[a]	2.30 ± 0.05[b]	2.73 ± 0.06[ab]	3.59 ± 0.1[a]
Total *cis* MUFA	20.99 ± 0.21[a]	47.76 ± 0.67[b]	42.15 ± 0.58[c]	36.52 ± 0.34[d]
18:1n-9	20.58 ± 0.26[a]	46.95 ± 0.54[b]	41.37 ± 0.59[c]	33.35 ± 1.11[d]
18:1 and other *cis* isomers[b]	0.41 ± 0.03 a	0.81 ± 1.01 a	0.78 ± 0.08 a	3.17 ± 0.1 b
Total PUFA	26.55 ± 0.97[a]	18.22 ± 1.41[bc]	19.82 ± 0.96[b]	14.14 ± 0.43[c]
C18:2 n-6	24.37 ± 0.55[a]	15.22 ± 0.48[c]	19.38 ± 0.65[b]	13.60 ± 0.47[c]
C18:3 n-3	1.64 ± 0.15[a]	2.38 ± 0.11[b]	0.44 ± 0.09[c]	0.54 ± 0.09[c]
C20:4 n-6	0.42 ± 0.02[a]	0.37 ± 0.03[a]	0.29 ± 0.01[b]	0.24 ± 0.01[b]
C20:5 n-3	0.04 ± 0.004[a]	0.102 ± 0.01[b]	0.026 ± 0.005[ac]	0.034 ± 0.005[ac]
C22:6 n-3	0.08 ± 0.012[a]	0.150 ± 0.021[b]	0.036 ± 0.002[c]	0.042 ± 0.004[c]
Total *trans*	—	—	—	5.74 ± 0.14[b]
Total MUFA- *trans*	—	—	—	5.02 ± 0.52[b]
Total PUFA- *trans*	—	—	—	0.72 ± 0.03[b]

[a]Values are mean ± SEM for six animals per group. Different superscript letters indicate significant differences from one another at $P \leq 0.05$ as determined by Duncan's test. For abbreviations, see Table 1.
[b]Includes all positional cis-isomers of 18:1 except 18:1n-9.

the type of dietary fat. The proportion of SFA was higher in the PO group than in the other groups. The content of MUFA was significantly higher in the CO group than in the SO, PHVO, and PO groups. Higher levels of linoleic acid (18:2n-6) were detected in EPI of the SO group compared with that of the remaining groups. It is relevant to note that tFA were only incorporated into EPI lipids of the PHVO group, which received a diet rich in these isomers. When the essential PUFA were evaluated, a significant decrease was observed in the percentage of linoleic and linolenic (18:3n-3) FA in EPI of PHVO and PO groups compared with SO and CO groups. It can also be observed that the amount of longer-chain metabolites of n-3 and n-6 PUFA—such as arachidonic acid (AA), EPA, and DHA—was less than 1% of the total lipid in the EPI of all groups. However, the AA levels in adipose tissue of all dietary groups were (slightly) higher than EPA and DHA. Both PO and PHVO groups had lower AA and DHA levels in EPI than SO and CO groups. Moreover, DHA and EPA were more abundant in the CO group than in the other three groups.

The fat content and LR in EPI on the 45th day of postnatal life are listed in Table 4. The results show that the PO and PHVO diets increased the relative weight of EPI, the LR, and the lipid content in this tissue, in comparison with the SO and CO groups. Moreover, the PO group had a significant increase in LR and in lipid content in EPI compared with PHVO.

The protein content (mg/100 mg) of EPI was not affected by the different experimental diets (SO: 21.88 ± 0.51; CO: 21.16 ± 0.81; PO: 23.34 ± 0.84; PHVO: 23.70 ± 0.71). As well, no significant differences in LPL activity (μM/min/g protein) were observed among the dietary groups (SO: 237.12 ± 8.65; CO: 234.08 ± 7.50; PO: 244.70 ± 6.77; PHVO: 245.25 ± 11.56).

Table 5 shows the mean plasma concentrations of TC, HDL cholesterol, TAG, and glucose according to diet. TAG level was significantly lower in the PO group compared with the other groups. Total cholesterol was significantly higher in the PHVO group than the PO group, which showed a lower concentration of TC than the CO group. HDL cholesterol of the PHVO group was lower than that of the remaining groups. Plasma glucose concentration was similar for the various experimental groups.

TABLE 4
Weight, Lipid Content, and Lipogenesis Rate in Epididymal Adipose Tissue[a]

Epididymal White Adipose Tissue	SO	CO	PO	PHVO
Weight (g)	1.48 ± 0.06[a]	1.38 ± 0.08[a]	2.46 ± 0.07[b]	1.34 ± 0.04[a]
Lipid content (g/100 g tissue)	73.62 ± 0.48[a]	75.39 ± 0.87[a]	93.71 ± 1.34[b]	82.19 ± 1.71[c]
Lipogenesis rate (μmol ^3H_2O incorporated into lipid (g fresh tissue)$^{-1}$ h^{-1})	2.61 ± 0.38[a]	2.75 ± 0.94[a]	7.04 ± 0.64[b]	4.85 ± 0.49[c]

[a]Values are mean ± SEM for six animals per group. Different superscript letters indicate significant differences from one another at $P \leq 0.05$ as determined by Duncan's test. For abbreviations, see Table 1.

TABLE 5
Profile of Lipoproteins and Glucose[a]

Variable (mg/dL)	SO	CO	PO	PHVO
Total cholesterol	62.78 ± 2.68[ab]	68.65 ± 1.35[a]	60.06 ± 2.40[b]	70.15 ±2.49[a]
HDL cholesterol	37.49 ± 3.26[a]	38.97 ± 3.55[a]	44.89 ± 5.18[a]	28.62 ± 1.97[b]
TAG	77.83 ± 3.53[a]	84.56 ± 5.29[a]	48.39 ± 2.04[b]	88.24 ± 7.75[a]
Glucose	84.60 ± 7.45	88.75 ± 5.34	71.43 ± 3.03	70.29 ± 4.39

[a]Values are mean ± SEM for six animals per group. Different superscript letters indicate significant differences from one another at $P \leq 0.05$ as determined by Duncan's test. For abbreviations, see Table 1.

DISCUSSION

In the present study, we documented that diets rich in palmitic acid (PO) or *trans* isomers (PHVO), in spite of being normolipidic diets, caused greater body weight gain at a young age (14 and 45 d) than those rich in PUFA (SO) or MUFA (CO). The literature shows controversial results concerning the relationship between the intake of palm oil—a saturated fat—and obesity. Loh *et al.* (18) showed that body weight gain and carcass lipid content were greater in obese rats fed the high-fat palm olein diet than in those fed soybean diet. In addition, Lemmonnier *et al.* (19) reported that both epididymal and perirenal depots had more fat cells in male rats fed high-fat diets with palm oil than in those fed corn oil. Moreover, Shillabeer and Lau (20) demonstrated that diets rich in saturated fat promoted adipocyte replication. In this context, an understanding of the ability of FA to regulate factors such as adipocyte size and number, or lipid metabolism (adipogenesis and fat oxidation), particularly in young animals, would be of great interest to better understand human obesity.

In humans, long-term tFA intake has been shown to positively correlate with body mass index (21). In cells cultured in the presence of tFA, some studies demonstrated a lower fat accumulation in adipocytes (22). On the other hand, adult male rats fed a hyperlipidic diet containing partially hydrogenated fat showed no significant difference in body weight (23). Although these studies correlate the increase of adiposity with the type of FA consumed, all of the diets tested were hyperlipidic. An original aspect of the present study is that all diets were normolipidic, differing only in the FA sources. Thus, some of the discrepancies between the present study and the literature can be explained by differences in important variables such as the overall amount of fat in the diet, the age of the animals at the beginning of the study and the feeding period.

Alterations in dietary fat type have been shown to influence membrane composition, function, and metabolic processes in many tissues (24). Changes in body weight and carcass fat content of PHVO and PO groups may also be due to differences in energy retention as a result of changes in metabolism or food intake. Immediately after weaning, we observed that the PO group presented the highest food intake, whereas the PHVO had the lowest, although during the final period of the study no significant differences were detected among the groups. It is interesting to note that the differences in food intake observed in the PHVO group are opposite to the ones observed in body weight, suggesting that the higher body weight in this group is due to differences in energy retention, probably due to a greater metabolic efficiency, particularly during the perinatal period, which involves several metabolic and physiological adaptations (25).

There is little definitive information on the effects of the type of dietary fat on lipid metabolism of adipose tissue. We observed an increase in *de novo* synthesis of FA in the epididymal adipose tissue of the PHVO and PO groups, and there was more fat in this tissue in comparison with the other groups. Moreover, the rate of FA synthesis in the PO group was approximately 45% higher than in PHVO. This means that different dietary FA will have different effects on *de novo* FA synthesis in adipose tissue in the young rats, which might contribute to their selective storage in adipose tissue as well as to the development of obesity. On the other hand, if different types of dietary fat affect FA synthesis in adipose tissue in different ways, it would be interesting to know how the body metabolizes specific FA, including differences in their propensity for direct incorporation into adipose tissue triglyceride, whether they must first be metabolized prior to deposition, and whether they are oxidized at different rates or mobilized from fat cells more readily than others (26). These seem to be relevant issues, which may partly explain some conflicting results of the present study regarding the increase in *de novo* FA synthesis and in the total lipid content of EPI in the PO group.

Adipose tissue lipoprotein lipase (LPL) plays an important role in recruitment of circulating fat into adipocyte stores (27). In the present study, no significant differences were observed in the LPL activity of the epididymal adipose tissue among the different groups. Similar results were reported by Takeuchi *et al.* (28) in a study with rats fed diets containing different types of lipids (saturated and unsaturated). Thus, these results indicate that the increase in adiposity of PO and PHVO groups was not due to greater uptake by the adipose tissue of FA coming from the diet in the form of chylomicrons, or released by the liver into the blood, in the form of VLDL.

Upon evaluating the FA composition of EPI, we observed that it reflects primarily the specific FA content of the experimental diet. Our results on FA profile in adipose tissue are in agreement with previous reports (23,29). The amount of tFA incorporated into adipose tissue of animals fed partially hydrogenated fat (PHVO) follows the dietary FA profile, because these FA are not synthesized by nonruminant animals (23). Furthermore, it has been reported that the composition of adipose tissue is a suitable biomarker of dietary FA intake, particularly for tFA, n-3, and n-6 FA (30).

In the present study, groups PO and PHVO presented lower levels of AA (20:4n-6) and DHA (22:6n-3) in EPI than groups SO and CO. EPA and AA are both precursors of eicosanoids, which have been demonstrated to be related to adiposity (26,31). Recently, prostaglandins I2 and J2 have been shown to induce adipocyte differentiation (32,33), whereas prostaglandins E2 and F2α are described as inhibitors of the adipogenic process (33,34). Moreover, prostaglandin E2 produced in adipose tissue may affect lipolysis (34).

Studies suggest that dietary FA have a significant impact on the circulating lipid levels. Our findings confirm previous studies conducted by other authors who observed the harmful effects of hydrogenated fat on the increase in total cholesterol levels, and a decrease of the circulating HDL levels (35,36), as well as beneficial effects of palm oil on the circulating lipid levels (37). In humans, the effect of feeding palm oil on serum cholesterol was similar to that observed when feeding olive oil (38) or canola oil (39). Various hypotheses have been formulated to attempt to explain the hypocholesterolemic effect of palm oil. One proposal take into account the high proportion of MUFA in palm oil, which has been associated with improvements in the lipoprotein profile (40). Another proposal attributes it to the presence in palm oil of natural antioxidants such as tocotrienols, vitamin E, and carotenoids (37).

In the present study no significant differences among groups were found in LPL activity in the adipose tissue. However, a reduction in circulating TAG was found in the PO group, in comparison with the other groups. At this point we note that there is evidence that the metabolic effects of dietary TAG depend not only on their FA composition but also on the stereochemical configuration of specific FA in the TAG molecule (41). Diets containing palmitic acid in the *sn*-2 position have caused higher plasma TAG values than diets containing palmitic acid in the *sn*-1/3 positions both in rats (41) and in human infants (42). Palm oil contains about 40% palmitic acid, but only about 3% of it is in the *sn*-2 position. This could explain the reduced blood TAG levels observed in the PO group.

The hypotriglyceridemic effect of palm oil may also be related to the rate of oxidation. Leyton *et al.* (43) fed rats FA of different chain lengths and degrees of saturation and reported varied oxidation rates, with unsaturated FA showing higher oxidation rates than SFA. Although more direct experiments are required, it is possible that the palm oil FA, due to its higher proportion of unsaturated FA chains in position 2 of the TAG molecule (41), can be quickly oxidized, resulting in reduced TAG levels in rats fed a palm oil diet.

In conclusion, our study shows a difference in effect on lipid metabolism when various dietary fats are given to male rats during lactation and up to 45 d. The results suggest that an unsaturated FA-rich diet promotes less body weight gain and fat deposition than an SFA-rich diet; a diet rich in tFA increases plasma concentrations of cholesterol and decreases HDL cholesterol; and a diet rich in palmitic FA decreases TAG levels, whereas both palmitic acid and tFA-rich diets raise the lipogenesis rate in epididymal adipose tissue, leading to increased fat content of this tissue and increased body weight, these effects being more pronounced in rats fed a palmitic FA-rich diet.

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Mice Raised on Milk Transgenically Enriched with n-3 PUFA Have Increased Brain Docosahexaenoic Acid

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ABSTRACT: The brain contains high levels of the long-chain n-3 FA DHA(22:6n-3), mainly in the gray matter and synaptosomes. Adequate intake of DHA is crucial for optimal nervous system function, particularly in infants. Supplementation of infant formulas with DHA at levels similar to human breast milk is recommended for biochemical and functional benefits to neonates. We generated transgenic mice that produce elevated levels of n-3 PUFA in their milk by expressing the *Caenorhabditis elegans* n-3 FA desaturase under the control of a lactation-induced goat beta-casein promoter. To examine the postnatal effects of consuming the n-3-enriched milk, we compared the growth and brain and plasma FA composition of mouse pups raised on milk from transgenic dams with those observed for pups raised on milk from nontransgenic dams. A significant decrease in arachidonic acid (ARA, 20:4n-6) and concomitant increases in n-3 PUFA were observed in the phospholipid fraction of transgenic mouse milk. The n-6:n-3 FA ratios were 4.7 and 34.5 for the transgenic and control milk phospholipid fractions, respectively. DHA and DPA (22:5n-6) comprised 15.1% and 2.8% of brain FA from weanling mice nursed on transgenic dams, as compared with 6.9% and 9.2% for weanling mice nursed on control dams, respectively. This transgenic mouse model offers a unique approach to disassociate the effects and fetal programming resulting from a high n-6:n-3 FA ratio gestational environment from the postnatal nutritional effects of providing milk with differing n-6:n-3 FA ratios.

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The long-chain n-3 FA DHA (22:6n-3) is an essential component in neuronal tissues (1). Results from numerous studies have demonstrated the involvement of DHA in the normal function and development of the central nervous system (2–4). Mammals are unable to synthesize n-3 FA *de novo,* hence DHA or its 18-carbon-chain precursor, alpha-linolenic acid (ALA, 18:3n-3), must be obtained from dietary sources. Adequate intake of dietary DHA is crucial, particularly in infants, because data indicate that conversion of ALA to DHA by the endogenous desaturation-elongation pathway may not be sufficient to support the needs of growing infants (5,6). Human breast milk contains 0.05–1.4% DHA of total FA by weight, depending upon the maternal diet and stage of lactation (7). Until 2002, infant formulas in North America did not contain preformed DHA, but rather contained n-3 FA in the form of ALA (8).

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Abbreviations used: ALA, alpha-linolenic acid; ARA, arachidonic acid; LA, linoleic acid, LC-PUFA, long-chain PUFA.

Different sources of preformed DHA are currently available to supplement infant formula: TG extracted from algae or fish, and phospholipids extracted from egg yolks. The parent molecule that is used to deliver FA in the diet can have important effects on the extent of FA bioavailability and transport to specific tissues (9). TG structure, that is, the FA position on the glycerol molecule, affects digestion, absorption, and FA metabolism. Likewise, phospholipid species and FA profile can influence gastric lipase activity, plasma accretion, and tissue distribution (9,10). The acyl lipids in milk fat comprise a large number of different FA and give rise to one of the most complex naturally occurring fats. In human milk, long-chain PUFA (LC-PUFA) are more highly concentrated in phospholipids, so that although phospholipids make up only 0.08% of total milk fat, they carry 15% of LC-PUFA. The remaining 85% of LC-PUFA are in the form of TG (11). If a given lipid class, species, or structure is a more available source of DHA for developing neural structures, this would have important nutritional, economic, and industrial implications for the supplementation of infant formulas.

The nematode *Caenorhabditis elegans* is able to synthesize ALA by virtue of an endogenous n-3 FA desaturase that recognizes a range of 18- and 20-carbon n-6 substrates (12) (Fig. 1). Unlike mammalian desaturases that act on FA esterified to coenzyme A, the *C. elegans* n-3 desaturase is hypothesized to be an acyl-lipid desaturase, meaning that it desaturates lipid-bound FA (13). Acyl-lipid desaturases from lower

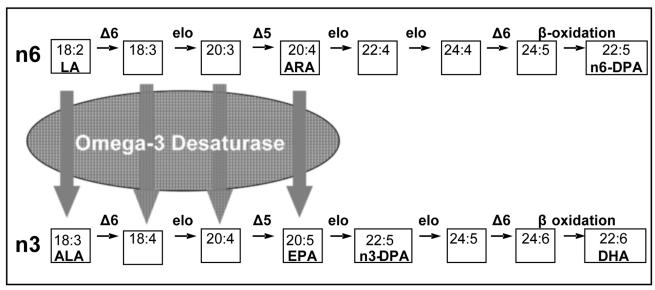

FIG. 1. Biosynthetic pathways of n-6 and n-3 LC-PUFA in mammals. FA elongases (elo) and desaturases (Δ5, Δ6) are capable of acting on either n-6 or n-3 FA to convert linoleic acid (LA) and alpha-linolenic acid (ALA) into LC-PUFA of the n-6 and n-3 families, respectively. The *C. elegans* n-3 FA desaturase recognizes a range of 18- and 20-carbon n-6 substrates. The synthesis of n-6- DPA and DHA requires a chain shortening brought about by one cycle of ,-oxidation in the peroxisome. ARA = arachidonic acid, EPA = eicosapentaenoic acid, DPA = docosapentaenoic acid, DHA = docosahexaenoic acid.

plants and microorganisms operate on acyl groups that are esterified to phospholipids, predominately at the *sn*-2 position (14). Constitutive expression of the *C. elegans* n-3 desaturase under the control of the chicken beta-actin promoter in transgenic mice was found to significantly increase the proportion of n-3 PUFA in total lipids of muscle, milk, brain, heart, erythrocytes, liver, lung, and spleen relative to the control mice (15).

We were interested in directing the expression of this transgene to the mammary gland during lactation as an approach to produce milk with elevated levels of n-3 FA. Transgenic mice expressing the *C. elegans* omega-3 desaturase under the control of the goat beta-casein lactation-induced mammary gland promoter were generated previously. This promoter has been found to direct transgene expression primarily in the lactating mammary gland of transgenic mice (16). These mice produce milk with elevated levels of n-3 FA, especially ALA and EPA (20:5n-3), with the most significant increase being observed in the LC-PUFA–rich phospholipid fraction (17). Several studies have suggested that the balance between n-6 and n-3 PUFA during the perinatal development has long-lasting effects on a number of metabolic parameters into adulthood (18–20). n-6 PUFA promote adipogenesis, and the increasing ratio of n-6:n-3 FA in the Western diet, particularly during fetal life and infancy, may be associated with increased adipogenesis and subsequent obesity (21). This transgenic model provides a way to transgenically decrease the n-6:n-3 FA ratio of milk from animals fed a high n-6:n-3 diet, and disassociate postnatal nutrition effects on programming (22), from those that result from a high n:6:n-3 gestational environment.

In this study we placed n-3 desaturase transgenic and control female mice on a high–n-6 FA diet at breeding and examined the neonatal growth and FA composition of their offspring after being raised on either control milk or milk transgenically enriched with n-3 FA.

TABLE 1
Composition and Calculated Analysis of High Safflower Experimental Diet (Diet D03092902, Research Diets, New Brunswick, NJ).

Ingredient	Amount in diet (g/kg)
Casein	211.7
DL-Methionine	3.2
Corn Starch	159.0
Sucrose	424.3
Cellulose, BW200	52.9
Safflower Oil	99.5
Mineral Mix S10001[a]	37.0
Vitamin Mix V1001[b]	10.6
Choline Bitartrate	2.1
Calculated analysis[c]	
Protein (g/100 g)	21
Carbohydrate (g/100 g)	59
Fat (g/100 g)	10
kcal/g	4.13
Observed (g/100 g oil)	
12:0	0.11
14:0	0.48
16:0	9.30
16:1	0.24
18:0	3.16
18:1	16.79
18:2 (LA, n-6)	69.20
18:3 (ALA, n-3)	0.17
20:4 (ARA, n-6)	0
22:6 (DHA, n-3)	0
n-6:n-3	407

[a]100 g of mineral premix contained 14.9 g Ca, 11.4 g P, 1.4 g Mg, 10.3 g K, 0.9 g S, 2.9 g Na, 4.6 g Cl, 5.7 mg Cr, 17.1 mg Cu, 0.6 mg I, 128.6 mg Fe, 168.6 mg Mn, 0.5 mg Se, and 82.9 mg Zn.
[b]100 g of vitamin premix contained 40,000 IU vitamin A, 10,000 IU vitamin D3, 500 IU vitamin E, 5 mg menadione sodium bisulfite (62.5% menadione), 2 mg biotin (1%), 100 µg cyancocobalamin (0.1%), 20 mg folic acid, 300 mg nicotinic acid, 160 mg calcium pantothenate, 70 mg pyridoxine HCl, 60 mg riboflavin, and 60 mg thiamin HCl.
[c]Calculated analysis provided by Research Diets, New Brunswick, NJ.
LA, linoleic acid; ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid.

EXPERIMENTAL PROCEDURES

Transgenic mice. Transgenic mice (C57/BL6 × DBA) were generated at the UC Davis Murine Targeted Genomics Laboratory by pronuclear microinjection of the *C. elegans* omega-3 FA desaturase (GenBank accession number L41807) under the control of the goat beta-casein promoter of the pBC1 mammary expression vector (Invitrogen, Carlsbad, CA) as detailed in Kao *et al*. (17). The animal experimentation was carried out in accordance with the regulations of the American Association for Accreditation of Laboratory Animal Care in fully accredited facilities at Cole B Vivarium of the UC Davis Department of Animal Science. Eight heterozygous transgenic and eight null (non-transgenic) full-sib control females were placed on a high-safflower mouse diet (Table 1, Diet D03092902, Research Diets, New Brunswick, NJ) at pairing with C57/BL6 males and maintained on this diet for the duration of the study. Pups were weaned to the experimental diet of the dam. Thirty-three pups from six litters per lactational group (ranging from 2 to 8 pups per litter) were nursed on these dams and weighed at days 10 and 21 postpartum. Toe clips from potential transgenic pups were added to 100 µL of lysis buffer [10 mM Tris-HCl, pH 8.0, 50 mM KCl, 20 g proteinase K (EM Science, Darmstadt, Germany), 0.5% Nonidet P-40 substitute (Calbiochem, San Diego, CA), and 0.5% Tween] and incubated at 55°C for 3 h, agitating every 1 h. Toe samples were then heated to 95°C for 10 min to denature the proteinase K, and DNA in the lysate was used as a template for PCR using the n-3 desaturase forward and reverse primers (5′GTCGACCATGGTCGCTCATTCCTCAG3′; 5′GTCGACTTACTTGGCCTTTGCCTTC3’). Transgenic pups were identified by the presence of a 1,444-bp PCR product.

Milk collection and analysis. Duplicate milk samples were collected from the 16 dams during peak lactation (days 10 and 12 postparturition). Phospholipid (polar lipids) and TAG (nonpolar lipids) fractions of the milk fats were separated using Sep-pak silica cartridges WAT051900 (Waters, Milford, MA). The TAG fraction was eluted with hexane:ethyl ether (1:1, vol/vol), and the phospholipid fraction was eluted with

methanol and chloroform:methanol:water (3:5:2, by vol) according to Bitman *et al.* (23). Both fractions were methylated by adding 10% acetyl chloride and incubated at 100°C for 1 h, followed by the addition of 6% K_2CO_3 and hexane. The samples were centrifuged for 5 min at 1,800 rpm, and the upper solvent layer was collected, evaporated under nitrogen, and resuspended in hexane (17). The resulting FAME were then resolved by GC (Hewlett Packard 5890 gas chromatograph). FA were identified by comparison of retention times to known standards (NuChek Prep, Elysian, MN) including 18:1, (18:2, 20:4, 22:5) n-6 FA, and (18:3, 20:5, 22:5, 22:6) n-3 FA, and expressed as g/100 g FA.

Brain and plasma collection and analysis. Whole brains were harvested from pups at 0 d (n = 7 from a control dam), 12 d (n = 7 per lactational group), 21 d (n = 33 per lactational group), and 42 d (n = 7 per lactational group). Brain samples were stored at –80°C until further analysis. For plasma collection, whole blood was collected by cardiac puncture of 21 d pups (n = 33 from 6 litters per lactational group). Blood samples from all pups in each litter were pooled to obtain adequate plasma for FA analysis and placed in a 1.5-mL tube containing 20 μL of 0.5 M EDTA to prevent blood coagulation. To separate the plasma from the erythrocytes, whole blood samples were centrifuged at 3,000 rpm at 4°C for 10 min, and plasma was stored at –20°C until further analysis.

To extract the total lipids, brain samples were homogenized with 0.5 mL of phosphate buffered saline. The brain homogenate samples were methylated with 2 mL 2% H_2SO_4 in methanol at 90°C for 30 min, and the solution was cooled to room temperature and neutralized with 10 mL of 6% K_2CO_3. The samples were vortexed, and 3 mL of hexane and 1 mL of water were added to the solution. The samples were vortexed again and centrifuged at 1,800 rpm for 5 min. The top organic layer was removed and evaporated under nitrogen and resuspended in 100 μL of iso-octane. Total lipids from plasma samples were methylated using the same method, and FA composition was analyzed by GC as previously described (17).

Statistical analysis. Values reported are means ± SEM. Milk, brain, and plasma FA composition and pup weight data analyses were performed using SAS statistical analysis software (SAS Institute, Cary, NC). The procedure MIXED was used to perform an ANOVA to test for differences between data from transgenic and control treatment groups. The differences between the DHA, n-6 DPA (22:5n-6), and total 22-carbon polyene (DHA + DPA) brain FA content of the pups nursed on transgenic dams over time (days 0, 12, 21, and 42 postpartum) were analyzed using a two-way ANOVA. Differences were considered significant if $P < 0.05$.

RESULTS

Milk FA analysis. The FA profiles of TAG and phospholipid fractions of milk produced by n-3 desaturase transgenic dams (n = 8) were compared with those from non-transgenic full-sib control (n = 8) dams when fed a high–linoleic acid (LA, 18:2n-6) diet (Table 2). A significant decrease ($P < 0.001$) in ARA and increases in the n-3 PUFA ALA ($P < 0.001$), EPA ($P < 0.001$), and DHA ($P < 0.05$) were observed in the phospholipid fraction of milk. The n-6:n-3 FA ratios were 4.7 and 34.5 for the transgenic and control milk phospholipid fractions, respectively. In the milk TAG fraction, a significant decrease was observed in ARA ($P < 0.01$), and increases were observed in EPA ($P < 0.001$) and n-3 DPA ($P < 0.01$). The n-6:n-3 FA ratios were 32.2 and 43.5 for the transgenic and control milk TAG fractions, respectively.

Pup plasma FA analysis. The blood from 21-d-old pups nursed on transgenic or non-transgenic full-sib dams was pooled within litter, and plasma FA composition was analyzed (Table 2). The n-6 DPA level was decreased ($P < 0.001$) and n-3 PUFA (EPA, n-3 DPA, and DHA) and stearic acid (18:0) levels were increased ($P < 0.01$–0.001) in plasma from pups nursed on transgenic dams.

Pup brain FA analysis. The FA composition of total brain lipids from 21-d-old pups nursed on n-3 desaturase transgenic and non-transgenic full-sib control dams were analyzed by GC (Table 2). Decreased ($P < 0.001$) levels of myristic acid (14:0), palmitic acid (16:0), ARA, and n-6 DPA (22:5n-6), and increased levels ($P < 0.01$–0.001) of ALA, EPA, n-3 DPA, and DHA were observed in the brains of 21-d-old pups nursed on transgenic dams, compared with the pups nursed on control dams. Twelve-day-old pups nursed on transgenic dams also showed less stearic acid, ARA, and n-6 DPA ($P < 0.01$–0.001) in their brains, and more DHA ($P < 0.01$) as compared with control-reared pups (Table 2). A rapid increase in the proportion of DHA and a decrease in n-6 DPA were observed over time in the brains of pups raised on transgenic dams. In contrast, the proportion of n-6 DPA in the brain increased over time in the pups raised on non-transgenic dams (Fig. 2). There was no effect of pup genotype (heterozygous versus full-sib null) on pup brain FA composition derived from pups nursed on heterozygous transgenic females (data not shown).

Pup weight analysis. The average weight of pups nursed on n-3 desaturase transgenic dams, 4.66 ± 0.11 g and 6.50 ± 0.19 g at day 10 and day 21 postpartum, respectively, did not differ from the average weight of pups nursed on control dams, 4.78 ± 0.17 g and 7.35 ± 0.15 g at day 10 and day 21 postpartum, respectively. Transgenic pups weighed an average of 4.30 ± 0.14 g and 6.23 ± 0.35 g at day 10 and day 21 postpartum, respectively, and this did not differ significantly from the average weights of nontransgenic pups, 4.73 ± 0.11 g and 6.77 ± 0.14 g.

DISCUSSION

Expression of the n-3 desaturase in the mammary gland had a significant effect on the FA composition of milk lipids in agreement with previous work (17). This effect was most striking in the milk phospholipid fraction, where there were 5-, 23-, and 3-fold increases in ALA, EPA, and DHA, respectively, relative to milk phospholipids from nontransgenic controls on the same

TABLE 2
FA Compositions of Milk Phospholipids (PL) and TG Derived from n-3 Desaturase Transgenic and Control Dams, and Those Measured in Blood Plasma and Brain Lipids from Their Nursing Offspring[a]

	N	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2n-6
Dam Milk							
Control PL	16	2.33 (0.44)	2.64 (0.71)	20.18 (0.83)	22.55 (1.21)	10.92 (0.45)	25.88 (1.00)
Transgenic PL	16	2.38 (0.47)	2.72 (0.76)	20.16 (1.00)	21.92 (1.21)	11.74 (0.39)	24.22 (0.71)
Control TG	16	9.52 (0.11)	11.43 (0.12)	21.16 (0.19)	2.11 (0.03)	16.50 (0.15)	26.68 (0.56)
Transgenic TG	16	9.35 (0.09)	11.48 (0.19)	21.11 (0.16)	1.99 (0.03)	16.48 (0.19)	27.17 (0.40)
Pup 21d Plasma							
Control Dam	6	0.71 (0.17)	1.00 (0.21)	24.80 (1.08)	16.42 (1.30)	6.97 (0.36)	29.49 (0.42)
Transgenic Dam	6	0.65 (0.11)	0.65 (0.16)	22.87 (1.18)	19.41[d] (1.37)	6.88 (0.42)	27.57 (0.29)
Pup 12d Brain							
Control Dam	7	0.02 (0.00)	1.62 (0.16)	25.51 (0.74)	19.06 (0.45)	13.70 (0.19)	1.26 (0.03)
Trasngenic Dam	7	0.15 (0.14)	1.56 (0.24)	25.59 (1.21)	17.45[b] (1.09)	12.88 (0.95)	1.21 (0.08)
Pup 21d Brain							
Control Dam	33	0	0.51 (0.02)	23.52 (0.20)	21.20 (0.10)	16.58 (0.06)	1.07 (0.01)
Trasngenic Dam	33	0	0.29[d] (0.02)	17.15[d] (0.43)	21.26 (0.06)	16.40 (0.15)	1.22 (0.02)
Pup 42d Brain							
Control Dam	7	0	0.23 (0.01)	21.96 (0.18)	21.39 (0.14)	19.44 (0.16)	1.11 (0.01)
Trasngenic Dam	14	0	0.21 (0.01)	21.56 (0.01)	21.65 (0.20)	20.35[c] (0.18)	1.13 (0.04)

[a]Values are means ± SEM.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

diet (Table 2). In the TG fraction of transgenic milk, the increases in EPA and n-3 DPA were 24- and 5-fold, respectively. These increases were accompanied by concomitant decreases in n-6 FA.

Weanlings (21 d) raised on milk from transgenic dams had elevated levels of EPA, n-3 DPA, and DHA in plasma lipids, and ALA, n-3 DPA, and DHA (~ 2-fold increase) in brain lipids (Table 2). Conversely, weanlings nursed on control dams had elevated levels of n-6 DPA in both plasma and brain lipids, despite the fact that the proportion of n-6 DPA in milk FA was not significantly different between milk derived from transgenic and control dams. Many other studies with n-3–deficient diets have reported that a decrease in DHA is accompanied by an increase in n-6 DPA, and the DHA:n-6 DPA ratio can be used as an indicator of n-3 FA deficiency (24). This ratio was 0.75 for the control pups' brains, as compared with 5.39 for the brains of pups nursed on transgenic dams. It has been demonstrated that n-6 DPA is not equivalent to DHA for optimum brain function (25,26), suggesting that the brain has a highly specific structural requirement for DHA. Phosphatidylserine is one of the major phospholipids in many eukaryotic membranes. Neuronal tissues are especially rich in phosphatidylserine, which may contain a high proportion (up to 35%) of DHA (27,28). The loss of DHA due to n-3 PUFA deficiency decreases the phosphatidylserine content in neuronal membranes, which could lead to alterations in membrane properties and adverse functional consequences (29), such as learning disturbances (30,31), olfactory discrimination deficits(32,33), and reduced visual acuity (34).

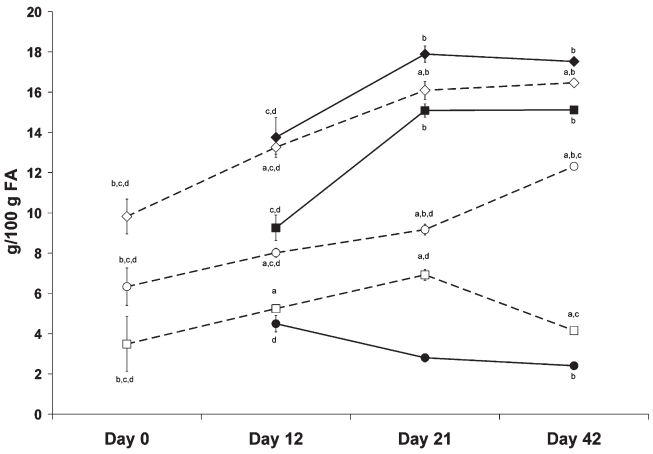

FIG. 2. Time course of 22-carbon polyene levels in the brains of young mice raised on milk from n-3 desaturase transgenic and control dams. DHA (□), n-6- DPA (○), and DHA + n-6 DPA (◇) g/100 g FA from pups ($n = 7$, 14, 66, and 21 at day 0, 12, 21, and 42, respectively) raised on milk from transgenic dams ($n = 8$; solid symbols and line) expressing an n-3 FA desaturase gene in the mammary gland during lactation, or from non-transgenic full-sib control dams ($n = 8$; open symbols and dashed line). Error bars represent SEM; a, b, c, and d denote significantly different ($P < 0.05$) from day 0, 12, 21, and 42 values for the same FA and treatment group, respectively.

The levels of DHA and n-6 DPA in the pup brains were divergent by 12 d postpartum (Fig. 2). An increase in pup brain DHA levels and a concomitant decrease in n-6 DPA levels were observed from 12 d postpartum until pups were weaned from transgenic dams (21 d). In pups raised on non-transgenic dams, the brain n-6 DPA level increased continuously over time, whereas the DHA level increased slowly until weaning and then decreased. Maternal body stores can be a significant source of DHA during lactation (8). The sum of DHA + n-6 DPA in the brains of pups raised on control dams fell behind

C18:3n-3	C20:4n-6	C20:5n-3	C22:5n-6	C22:5n-3	C22:6n-3	Sum n-6	Sum n-3	n-6/n-3
0.52 (0.48)	8.55 (0.69)	0.13 (0.44)	1.06 (0.15)	0.42 (0.25)	0.37 (0.29)	35.49	1.43	34.52
2.84^{d} (0.34)	4.96^{d} (0.49)	3.02^{d} (0.31)	0.81 (0.10)	0.64 (0.18)	1.20^{b} (0.20)	30.00	7.70	4.72
0.58 (0.10)	1.29 (0.08)	0.01 (0.02)	0.23 (0.03)	0.02 (0.02)	0.06 (0.01)	28.20	0.67	43.49
0.61 (0.07)	1.00^{c} (0.06)	0.24^{d} (0.02)	0.22 (0.02)	0.11^{c} (0.02)	0.07 (0.01)	28.39	1.03	32.23
0.14 (0.02)	13.23 (0.41)	0.02 (0.02)	3.62 (0.11)	0.06 (0.04)	1.02 (0.20)	46.34	1.23	39.29
0.10 (0.01)	12.88 (0.29)	0.18^{c} (0.01)	1.52^{d} (0.08)	0.51^{c} (0.03)	4.41^{d} (0.14)	41.96	5.19	8.55
0.003 (0.001)	17.44 (0.19)	0.01 (0.01)	8.02 (0.02)	0.13 (0.01)	5.24 (0.18)	26.79	5.24	5.13
0.002 (0.001)	15.12^{b} (0.06)	0.02 (0.01)	4.50^{c} (0.41)	1.03 (0.72)	9.25^{c} (0.64)	20.61	10.30	2.01
0.87 (0.04)	14.43 (0.17)	0.01 (0.001)	9.17 (0.02)	0.08 (0.01)	6.92 (0.26)	24.68	7.88	3.26
1.06^{d} (0.05)	14.00^{d} (0.27)	0.02^{c} (0.001)	2.80^{d} (0.21)	0.28^{d} (0.01)	15.09^{d} (0.33)	18.01	16.44	1.10
0	13.42 (0.05)	0	12.31 (0.08)	0.08 (0.004)	4.15 (0.11)	26.85	4.23	6.04
0	12.60^{d} (0.12)	0	2.41^{d} (0.09)	0.10^{c} (0.01)	15.12^{c} (0.19)	16.13	15.22	1.06

that of pups consuming transgenic milk during postnatal development. The "reciprocal replacement" of DHA by n-6 DPA in the brain that is associated with n-3 FA deficiency (35) is incomplete in young animals (36). Therefore, the total proportion of DHA and n-6 DPA 22-carbon polyenes in the brain declines when animals face n-3 FA deficiency during early development.

The inheritance of the transgene itself did not impact the brain FA, as evidenced by the fact that there was no significant effect of pup genotype (heterozygous versus null) in the statistical model (data not shown). This was predicted based on the nearly exclusive expression of the goat beta-casein promoter in the lactating mammary gland. Although discreet amounts of goat beta-casein expression have been observed in the skin and skeletal muscle of transgenic mice, no expression has been observed in the brain (16,17).

The molecular mechanism underlying the accretion of high concentrations of DHA in the brain is unknown (37). Despite the fact that the absolute levels of DHA and n-6 DPA relative to the total FA in milk were not greatly altered by transgenic expression of the n-3 FA desaturase, highly significant differences were observed in the FA composition of weanling brains. The brain rapidly accretes DHA during intrauterine growth and early postnatal "brain growth spurt." Afterward, the adult brain has been described as tenaciously retaining DHA, even during n-3 FA dietary deficiency (26,38). The continued divergence of brain DHA levels observed at day 42 (21 d after weaning) of 15.1% in transgenic-reared pups, as compared with 4.2% in control-reared pups, supports the contention that the brain retains DHA (Fig. 2). Mechanisms exist to minimize DHA loss from the brain during periods of n-3 FA deficiency (39), although it should be noted that the already low brain DHA levels in control-reared pups further declined from 6.9 to 4.2% in this post-weaning period.

Results from studies examining the effects of dietary source on PUFA availability and accumulation in the brain have been contradictory. Some researchers have reported that absorption (7) and brain accretion (37,40) of LC-PUFA are greater from phospholipid sources of LC-PUFA than TG sources, whereas others have concluded the reverse (41), or that the form of the dietary LC-PUFA does not influence their bioavailability (42,43). One explanation that has been put forward to address this apparent paradox is the questionable validity of extrapolating blood plasma FA composition data to estimate the essential FA status of the brain (37). It has been shown that, although plasma phospholipids are good indicators for liver and bile phospholipid FA status, they are not predictive of brain FA composition (37,44). Although our results agree with these findings in that there were no predictable associations between milk and pup plasma or brain FA compositions, this explanation does not resolve the fact that two animal studies found no effect of the form of dietary LC-PUFA on direct measurements of brain FA composition (45,46).

The weights of the pups raised on the transgenic milk were not significantly different from those of the pups raised on control milk, although there was a trend for the pups raised on control milk to be heavier. Other studies have shown that decreasing the n-6:n-3 FA ratio of milk fat, as was effected in the milk derived from the transgenic dams, decreases growth rate, reduces adipose tissue mass, and lowers serum leptin levels in nursing offspring (47,48).

The endogenous mammary gland production of n-3 FA in the transgenic mammalian model described in this study offers a

unique opportunity to examine the growth and development effects of decreasing the n-6:n-3 ratio of maternally-derived FA between gestation and lactation. It may be particularly valuable in isolating the effect of varying the n-6:n-3 FA ratio of milk on adipogenesis and the development of obesity. It is also well suited to answer questions related to the contribution of milk lipids to DHA accretion in the neonatal brain. The relative bioavailability of LC-PUFA associated with different species of milk phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine) could be examined, along with further exploration as to whether the form of LC-PUFA in milk impacts brain FA profiles in suckling neonates. This question has obvious practical relevance in that most infant formulas are currently being supplemented with an algal TAG source of n-3 LC-PUFA. This model may also be of utility to researchers working to further understand the effects of neonatal n-3 LC-PUFA nutrition on visual acuity, brain function, and behavioral development.

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Solubility in and Affinity for the Bile Salt Micelle of Plant Sterols Are Important Determinants of Their Intestinal Absorption in Rats

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ABSTRACT: Intestinal absorption of various plant sterols was investigated in thoracic duct–cannulated normal rats. Lymphatic recovery was the highest in campesterol, intermediate in brassicasterol and sitosterol, and the lowest in stigmasterol and sitostanol. Higher solubility in the bile salt micelle was observed in sitosterol, campesterol, and sitostanol than in brassicasterol and stigmasterol. The solubility of the latter two sterols was extremely low. When the affinity of plant sterols for the bile salt micelle was compared in an *in vitro* model system, which assessed sterol transfer from the micellar to the oil phase, the transfer rate was the highest in brassicasterol, intermediate in campesterol and stigmasterol, and lowest in sitosterol and sitostanol. Although no significant correlations between lymphatic recovery of plant sterols and their micellar solubility or transfer rate from the bile salt micelle were observed, highly positive correlation was obtained between the lymphatic recovery and the multiplication value of the micellar solubility and the transfer rate. These observations strongly suggest that both solubility in and affinity for the bile salt micelle of plant sterols are important determinants of their intestinal absorption in rats.

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Although plant sterols have similar chemical structures to cholesterol (Fig. 1), they are less absorbable than the latter in experimental animals and humans (1). It has been known that absorption rates of various plant sterols are also different from each other (1). However, precise mechanisms of their differential absorption are not understood in detail. Recently, Altmann *et al.* (2) and Davis *et al.* (3) reported that Niemann-Pick C1 Like 1 protein (NPC1L1) is involved in intestinal uptake of cholesterol and plant sterols. However, factors other than NPC1L1 may participate in sterol absorption, because about half of cholesterol and plant sterols was absorbed in NPC1L1-deficient mice compared with wild-type mice (2,3).

Another determinant for differential absorption of plant sterols is differences in efflux from the enterocyte by ATP-binding cassette transporter G5 (ABCG5) and ATP-binding cassette transporter G8 (ABCG8). ABCG5 and ABCG8 work as a heterodimer and secrete absorbed cholesterol and plant sterols from the enterocyte to the intestinal lumen (4). Several mutations of ABCG5 or ABCG8 were found in sitosterolemic patients, who deposit plant sterols in the body and have higher absorbability of plant sterols than normal subjects (5,6). There is a possibility that ABCG5 and ABCG8 might discriminate structural differences in various plant sterols. However, in ABCG5/ABCG8-deficient mice, differential absorption between sitosterol and campesterol was observed (7). Yu *et al.* also showed that when ezetimibe, a potential inhibitor of the NPC1L1 sensing pathway, was given to mice lacking ABCG5 and ABCG8, sterol absorption was reduced, but it was not negligible (8). These observations suggest that factors other than NPC1L1 and ABCG5/ABCG8 are also involved in intestinal absorption of plant sterols.

We previously showed a possibility that intestinal absorption of various plant sterols partly depend on their affinity for the bile salt micelle (9). Sitosterol and stigmasterol, less absorbable plant sterols, had higher affinity for the bile salt micelle than campesterol, which is more absorbable than the former sterols (9). Micellar solubilization of sterols is also essential for sterol absorption (10). Although some studies showed that the solubility of sitosterol in the bile salt micelle was lower than that of cholesterol (11,12), systematic studies by using various plant sterols have never been conducted.

In the present study, to reveal factors participating in plant sterol absorption, lymphatic absorption of various plant sterols in rats, as well as their micellar solubility and affinity for the bile salt micelle, were studied. In most of the studies on plant sterol absorption, plant sterol mixtures were given to animals and humans. Because each plant sterol inhibits intestinal absorption of other plant sterols, accurate comparison among their absorption is impossible. Therefore, we used purified plant sterols in the present study.

EXPERIMENTAL PROCEDURES

Materials. Brassicasterol (purity 93.5%), campesterol (94.4%), stigmasterol (97.7%), and sitosterol (98.6%) were kindly provided by Tama Biochemical Co., Ltd. (Tokyo, Japan). Cholesterol

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Abbreviations: ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; NPC1L1, Niemann-Pick C1 Like 1 protein.

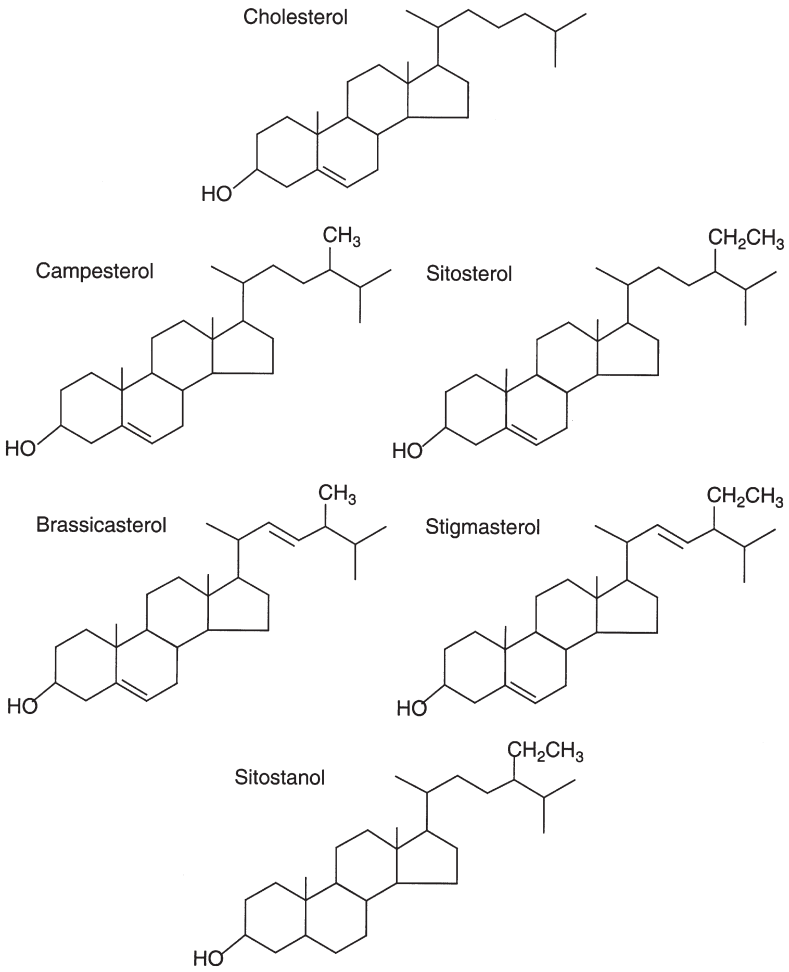

FIG. 1. Chemical structures of plant sterols and cholesterol.

(>99%), sitostanol (95.9%), oleic acid, 1-monooleoyl-rac-glycerol (1-monoolein), L-α-phosphatidylcholine, and triolein were purchased from Sigma (Tokyo, Japan). Sodium taurocholate (>97%) was purchased from Nacalai Tesque (Kyoto, Japan).

Lymphatic absorption of purified plant sterols in rats. Four-week-old male Wistar rats (n = 35) (Seac Yoshitomi, Fukuoka, Japan) were fed an AIN-93G purified diet containing 10% lard for 4 wk *ad libitum*. Because plant sterol content in lard was extremely low, plant sterol concentration in lymph of Wistar rats became almost negligible. These rats were subjected to surgery. Average body weight at surgery was 362 g. The left thoracic lymphatic duct cephalad to the cisterna chili was cannulated as described previously (13). A second indwelling catheter was placed in the stomach for administration of emulsion. After surgery, animals were placed in restraining cages and intragastrically given a continuous infusion of a solution containing 139 mM glucose and 85 mM NaCl at a rate of 3.4 mL/h until the end of experiment. The same solution was given as drinking water. The next morning, animals with a constant lymph flow rate were divided into five groups (n = 6–7 rats per group) to evaluate lymphatic absorption of different plant sterols. Lipid emulsions composed of 67 mg sodium taurocholate, 17 mg FA-free BSA fraction V (Serologicals, Kankakee, IL), 67 mg triolein, and 6.67 mg of a purified plant sterol in 1 mL were prepared by sonication and one of the emulsions was administered into the stomach for 1 mL at 100 g body weight. In this study, five types of purified plant sterols, specifically brassicasterol, campesterol, stigmasterol, sitosterol, and sitostanol, were used for preparing test emulsions. Lymph was collected on ice-chilled tubes containing EDTA for 24 h, and aliquots were served to sterol analysis. In a separate study, lymphatic recovery of cholesterol was also evaluated in the same experimental condition (n = 6, average body weight 303 g). Because cholesterol and trace amounts of plant sterols were detected in lymph before the infusion of a test emulsion, the amounts of cholesterol and plant sterols in lymph before administration were used as the baseline. All animal studies were carried out under the guidelines for animal experiments of the Faculty of Agriculture, Graduate School, Kyushu University, and Law 105 and Notification 6 of the government of Japan.

Micellization of purified plant sterols in vitro. Lipid emulsions containing 10.8 mM oleic acid, 10.8 mM triolein, 5.4 mM

TABLE 1
Lymphatic Absorption, Micellar Solubility, and Transfer Rate to Triolein of Purified Plant Sterols

	Sterols					
	Cholesterol[a]	Brassicasterol	Campesterol	Stigmasterol	Sitosterol	Sitostanol
Lymphatic absorption[b]						
Recovery (%)	59.2 ± 5.16	3.31 ± 0.42[a]	15.4 ± 1.34[b]	0.47 ± 0.06[c]	2.36 ± 0.33[ac]	0.30 ± 0.03[c]
Mass (μg/100 g body weight)	3,863 ± 337	134.1 ± 16.6[a]	918 ± 90.7[b]	25.9 ± 3.68[a]	125 ± 18.0[a]	15.2 ± 1.60[a]
Micellar solubility[c] (mM)	5.21 ± 0.07	0.52 ± 0.02[a]	2.10 ± 0.04[b]	0.48 ± 0.004[a]	2.28 ± 0.04[c]	1.78 ± 0.05[d]
Transfer rate[d] (%)	16.6 ± 1.62	15.4 ± 1.18[a]	8.03 ± 0.37[b]	6.34 ± 0.44[bc]	5.04 ± 0.42[cd]	3.19 ± 0.22[d]

[a]Data on cholesterol were obtained in separate studies. Therefore, the data were not subjected to statistical analyses.
[b]Data are mean ± SE of 6 to 7 rats per group.
[c]Data are mean ± SE of 3 determinations.
[d]Data are mean ± SE of 4 determinations.
[a,b,c]Different letters show significant differences among plant sterols at $P < 0.05$ in the Tukey-Kramer test.

1-monoolein, 6 mM sodium taurocholate, and 0.5 to 8 mM of a purified plant sterol, either brassicasterol, campesterol, stigmasterol, sitosterol, or sitostanol, were prepared in 150 mM sodium phosphate buffer (pH 7.0) by sonication. The emulsions thus obtained were kept at 37°C and transferred to a Beckman Quick Seal tube and ultracentrifuged at 100,000 × *g* for 1 h at 37°C (Optima TL Ultracentrifuge, Beckman, Palo Alto, CA) to separate the micellar phase from the emulsion. Aliquots of the clear micellar solution and the emulsion were served to sterol analysis by GLC. Micellar solubility of cholesterol was also examined at 8 mM in a separate study.

Transfer of purified plant sterols from the bile salt micelle to triolein. Micellar solutions containing 6.6 mM sodium taurocholate, 0.6 mM phosphatidylcholine, 0.1 mM of a purified plant sterol, either brassicasterol, campesterol, stigmasterol, sitosterol, or sitostanol, and 132 mM NaCl in 15 mM sodium phosphate buffer (pH 7.4) were prepared. The micelles then passed through a 0.2-μm syringe filter (25 mm, GD/X, Whatman, Inc., Clifton, NJ) and kept at 37°C overnight. The next morning, 1.5 mL of a micellar solution and 0.5 mL of triolein were placed in a plastic tube, flushed with N_2, and sealed. The tubes were incubated at 37°C in an oscillating water bath for 6 h (190 oscillations/min), at which time the rate of sterol transfer from the micellar solution to triolein was linear. At the end of the incubation, the contents of each tube were transferred to a Beckman Quick Seal tube, and the oil and aqueous phases were separated by ultracentrifugation (Beckman) at 100,000 × *g* for 1 h at 37°C. The oil and micellar phases were collected and sterol concentrations were analyzed by GLC (9). Transfer of cholesterol from the bile salt micelle to triolein was also examined under the same experimental conditions.

Sterol analysis. Total lipids were extracted and purified by the method of Folch *et al.* (14). After saponification of total lipids, unsaponifiable matters collected were derivatized to trimethylsilylethers and were quantified by GLC using a SPELCO SPB-1™ column (0.25 mm × 60 m, 0.25-μm film thickness, Sigma-Aldrich, Tokyo, Japan) and 5α-cholestane (Sigma) as an internal standard.

Statistics. All data were expressed as mean ± SE. The Tukey-Krammer test was performed, and $P < 0.05$ was considered significant.

RESULTS

Lymphatic absorption of purified plant sterols in Wistar rats. The lymphatic 24-h recovery of campesterol was the highest among the five types of plant sterols, and stigmasterol and sitostanol were the lowest (Table 1). Lymphatic recoveries of brassicasterol and sitosterol were intermediate. The lymphatic 24-h recovery of cholesterol was 59.2 ± 5.2% under the same experimental conditions.

Micellar solubility of purified plant sterols in vitro. Micellar concentrations of purified plant sterols proportionally increased with the increase of their concentration from 0.5 to 6 mM in test emulsions. The micellar concentration of each plant sterol reached plateau when the concentration in emulsion was 8 mM. Therefore, we thought that maximal solubility of each plant sterol was obtained at 8 mM in test emulsion under our experimental conditions. Among the five types of purified plant sterols, the solubility of brassicasterol and stigmasterol were extremely low (Table 1). Micellar solubilities of campesterol, sitosterol, and sitostanol were 3.5–4.5 times higher than those of brassicasterol and stigmasterol. Micellar concentration of cholesterol in the 8 mM emulsion was 5.21 ± 0.07 mM in a separate study.

Transfer of micellar plant sterols to triolein. We investigated the transfer rates of purified plant sterols from the bile salt micelle to triolein at 37°C *in vitro*. In this condition, it is thought that degrees of movement of micellar sterols to the oil phase depend on affinity of sterols for the bile salt micelle (9), that is, the higher the sterol transfer to triolein, the lower the affinity of the sterol for the bile salt micelle. The transfer rate of plant sterols was the highest in brassicasterol and the lowest in sitostanol among the five types of plant sterols tested (Table 1). The order of transfer rates of each plant sterol was as follows: brassicasterol > campesterol > stigmasterol > sitosterol > sitostanol. The transfer rate of cholesterol was comparable to that of brassicasterol.

Correlation between lymphatic recovery of sterols and their micellar solubility or transfer rate. There was no positive correlation between micellar solubility and lymphatic recovery of plant sterols ($r = +0.41$, $P = 0.50$), or between transfer rate of micellar plant sterols to triolein and lymphatic recovery of plant sterols ($r = +0.21$, $P = 0.74$). However, a

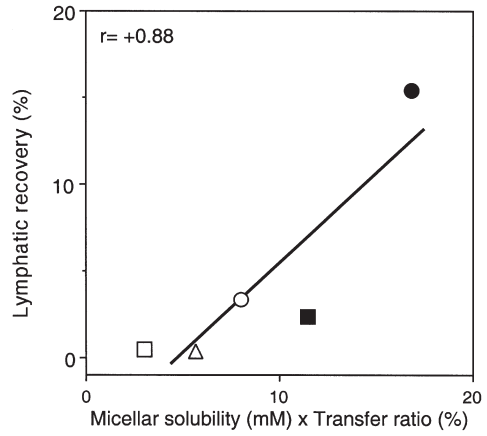

FIG. 2. Correlation between lymphatic recovery of plant sterols and multiplication value of micellar solubility and transfer rate of micellar sterol to triolein. ○, Brassicasterol; ●, campesterol; □, stigmasterol; ■, sitosterol; △, sitostanol.

highly positive correlation was observed (Fig. 2) between lymphatic recovery of plant sterols and multiplication value of micellar solubility and transfer rate ($r = +0.88$, $P < 0.05$). When data for cholesterol, collected in separate studies, were included, the correlation coefficient between these parameters was $r = +0.99$, $P < 0.001$.

DISCUSSION

In the present study, we measured absorption rates of sterols by the lymphatic recovery method. Absorption rates measured by the intestinal perfusion method and the fecal recovery must be lower than those measured in the lymphatic recovery method, because in intact animals, a part of absorbed sterols is excreted via bile into intestinal lumen and then into feces. Absorption rates excluding influence of biliary excretion of sterols may be important to consider the relationship between absorption rates of sterols and micellar solubility and affinity for the bile salt micelle of sterols.

We compared lymphatic absorption of various purified plant sterols in rats and showed that intestinal absorption rates of various plant sterols vary widely. Lymphatic 24-h recovery of campesterol was about 50 times higher than that of sitostanol, the least absorbable sterol (Table 1). The recovery of stigmasterol was almost comparable to that of sitostanol. Sitosterol and brassicasterol were more absorbable than sitostanol and stigmasterol, and less absorbable than campesterol.

We previously showed that fecal output of dietary sitostanol in rats was almost quantitative (15). When fecal output of ^{14}C-sitostanol was assessed in rats, more than 97% was recovered in feces (16). Hassan and Rampone showed that lymphatic absorption of trace amounts of ^{3}H-sitostanol in 24 h was 2.2% in rats (17). In the present study, lymphatic recovery of sitostanol was only 0.3%, and therefore it is clear that sitostanol absorption is extremely low.

It has been reported that sitosterol is more absorbable than sitostanol (16). Sitosterol absorption rate has been measured in many studies and reported to be 5–32% in humans and rats (18). According to relatively reliable observations, sitosterol absorption may be around 5% in rats and humans (19–23). Although intestinal absorption of campesterol has been suggested to be higher than sitosterol, only two previous studies showed preferential absorption of campesterol over sitosterol. Heinemann *et al.* reported that intestinal absorptions of campesterol and sitosterol were estimated to be 9.6% and 4.2%, respectively, in healthy humans measured by an intestinal perfusion technique (23). Lütjohann *et al.* showed that absorptions of campesterol and sitosterol were estimated to be 16% and 5% in normal subjects by measuring fecal output of deuterium-labeled sterols (22). However, there is no precise observation of campesterol absorption rate in rats. In the present study, higher absorbability of campesterol than sitosterol was proved in rats. Bhattacharyya measured uptake of campesterol and sitosterol by rat small intestine *in vivo* (24). The amounts incorporated into intestinal mucosa were higher in campesterol than in sitosterol.

The results on intestinal absorption of stigmasterol and brassicasterol are controversial. Heinemann *et al.* observed almost comparable absorption of stigmasterol to that of sitosterol in humans (23). Vahouny also observed lymphatic 24-h recoveries of stigmasterol and sitosterol were 4.3% and 3.1%, respectively, in rats (25). These observations suggest that absorption of stigmasterol is comparable to that of sitosterol. In contrast, Bhattacharyya showed extremely lower incorporation of stigmasterol than sitosterol into the small intestine in rat (24). In the present study, the rate of stigmasterol absorption was extremely low and comparable to that of sitostanol absorption. Vahouny showed that lymphatic absorption of brassicasterol included in oyster sterols was 5.4% for 12 h in rats (26). Gregg *et al.* reported that absorption of brassicasterol (given as shellfish sterols) was 4.8% in normal subjects (27). The values seem to be higher than the absorption rate of brassicasterol in the present study. Because studies on absorption of stigmasterol and brassicasterol are scarce, more research is necessary.

To clarify the involvement of micellar solubility and affinity for the bile salt micelle of various plant sterols, these parameters were measured. The micellar solubility of plant sterols increased in the following order: stigmasterol = brassicasterol < sitostanol < campesterol < sitosterol. There was no significant correlation between micellar solubility and lymphatic recovery of plant sterols ($r = +0.41$). The affinity of plant sterols for the bile salt micelle was assessed by measuring their transfer from the bile salt micelle to triolein. In our previous study, the transfer of sterols classified as absorbable (cholesterol, campesterol, 7-dehydrocholesterol) was higher than that of sterols known to be less absorbable (sitosterol, stigmasterol, fucosterol) (9). Therefore, we concluded that intestinal absorption of sterols depends on the affinity for the bile salt micelle. In the present study, when we reevaluated the transfer of five types of plant sterols, newly including brassicasterol and sitostanol, the transfer rate increased in the following order: sitostanol < sitosterol < stigmasterol < campesterol < brassicasterol. No positive correlation was observed between their transfer rate and lymphatic absorption ($r = +0.21$). In contrast

to our previous study, the results in the present study suggest that affinity to the bile salt micelle is not a sole determinant of plant sterol absorption. Hence, we thought that both micellar solubility and affinity for the bile salt micelle must relate to intestinal absorption of plant sterols. We multiplied amounts of plant sterols solubilized in the bile salt micelle by transfer rate from the bile salt micelle and found that there is a highly positive correlation between lymphatic absorption rate and the multiplicative product of micellar solubility and transfer rate (r = +0.88). When results obtained from separate experiments using cholesterol were included, correlation coefficient was r = +0.99. The observation strongly suggests that both micellar solubility and transfer rate are involved in differential absorption rate of plant sterols and cholesterol.

It has been reported that NPC1L1 is involved in intestinal uptake of cholesterol and plant sterols (2,3). Ezetimibe, a potential inhibitor of the NPC1L1 sensing pathway, reduced their absorption. Davis *et al.* reported that the uptake of sitosterol into the intestine of NPC1L1-deficient mice and normal mice given ezetimibe was reduced by around 50% (3). Yu *et al.* also reported that absorption of campesterol and sitosterol in normal mice was reduced by the administration of ezetimibe roughly to one-half (8). These observations suggest that 50% of incorporation of sterols into intestinal epithelial cells is dependent on the pathway through NPC1L1. We think that micellar solubility and affinity for the bile salt micelle participate in the remaining part (about 50%) of uptake of plant sterols into the intestine. However, we will have to examine a possibility that NPC1L1 contributes to differential absorption of plant sterols.

There is a possibility that ABCG5 and ABCG8 also participate in differential absorption of plant sterols. Igel *et al.* proposed a hypothesis that the uptake of sterols and their corresponding stanols into the intestinal mucosal cell are extremely rapid and unselective process, hence, discrimination takes place on the level of reverse transport back into the gut lumen by ABCG5 and ABCG8 (28). However, Yu *et al.* observed that although intestinal absorptions of cholesterol, campesterol, and sitosterol in AGCG5/ABCG8-deficient mice were higher than those in the wild-type mice, the rank order of absorption rate was cholesterol > campesterol > sitosterol in both mice (7). Therefore, they described that differential absorption of plant sterols is largely independent of ABCG5 and ABCG8 (7). Further investigation is necessary to clarify whether the rates of efflux of various plant sterols by ABCG5 and ABCG8 are different.

In the present study, we showed that solubility in and affinity for the bile salt micelle of plant sterols are important determinants of their intestinal absorption. The extent of contribution of these parameters, the incorporation by NPC1L1, and the efflux by ABCG5 and ABCG8 on plant sterol absorption remain to be solved.

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Diacylglycerol Acyltransferases from *Vernonia* and *Stokesia* Prefer Substrates with Vernolic Acid

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ABSTRACT: Genetic engineering of common oil crops for industrially valuable epoxy FA production by expressing epoxygenase genes alone had limited success. Identifying other key genes responsible for the selective incorporation of epoxy FA into seed oil in natural high accumulators appears to be an important next step. We investigated the substrate preferences of acyl CoA:diacylglycerol acyltransferases (DGAT) of two natural high accumulators of vernolic acid, *Vernonia galamensis* and *Stokesia laevis*, as compared with a common oilseed crop soybean. Developing seed microsomes were fed with either [^{14}C]oleoyl CoA or [^{14}C]vernoloyl CoA in combinations with no exogenous DAG or with 1,2-dioleoyl-*sn*-glycerol, 1-palmitoyl-2-vernoloyl-*sn*-glycerol, 1,2-divernoloyl-*sn*-glycerol, 1,2-dioleoyl-*rac*-glycerol, or 1,2-divernoloyl-*rac*-glycerol to determine their relative incorporation into TAG. The results showed that in using *sn*-1,2-DAG, the highest DGAT activity was from the substrate combination of vernoloyl CoA with 1,2-divernoloyl-*sn*-glycerol, and the lowest was from vernoloyl CoA or oleoyl CoA with 1,2-dioleoyl-*sn*-glycerol in both *V. galamensis* and *S. laevis*. Soybean DGAT was more active with oleoyl CoA than vernoloyl CoA, and more active with 1,2-dioleoyl-*sn*-glycerol when oleoyl CoA was fed. DGAT assays without exogenous DAG, or with exogenous *sn*-1,2-DAG fed individually or simultaneously showed consistent results. In combinations with either oleoyl CoA or vernoloyl CoA, DGAT had much higher activity with *rac*-1,2-DAG than with their corresponding *sn*-1,2-DAG, and the substrate selectivity was diminished when *rac*-1,2-DAG were used instead of *sn*-1,2-DAG. These studies suggest that DGAT action might be an important step for selective incorporation of vernolic acid into TAG in *V. galamensis* and *S. laevis*.

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Epoxy FA such as vernolic acid have many industrial applications including in plasticizers (1), coatings (2), and resins (3). A few plant species such as *Vernonia galamensis* (2) and *Stokesia laevis* (4) accumulate vernolic acid as their major FA in their seed oils. However these plants, in their present form, cannot readily be grown to produce seed efficiently on an industrial scale. Soybean and linseed oil are currently utilized to produce epoxidized oil using a chemical process. Epoxygenase genes that are responsible for epoxidizing linoleic acid while in the *sn*-2 position of phospholipids such as PC (5) have been cloned from several accumulators of vernolic acid (6–9). However, expressing *V. galamensis* epoxygenase in soybean seeds resulted in an accumulation of only 8% vernolic acid of the total FA of the seed oil (10), which is not high enough to be commercially viable. Similar results were found with arabidopsis seeds (9). Thus, other key enzymes in seed oil biosynthesis need to be identified in high accumulators of epoxy FA to achieve greater epoxy FA accumulation in transgenic oilseeds. Three enzymes, namely acyl CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), phospholipid:diacylglycerol acyltransferase (EC 2.3.1.158), and diacylglycerol transacylase, are known to be able to carry out the final step in TAG synthesis in developing seeds (11). These enzymes may play an important role for the selective incorporation of unusual FA into TAG in natural high accumulators of unusual FA. For example, the DGAT of castor (*Ricinus communis*), an accumulator of ricinoleic acid, has been shown to favor diricinoleoylglycerol and ricinoleoyl CoA incorporation into TAG (12–14).

In the present study we test the hypothesis that *V. galamensis* and *S. laevis* DGAT but not soybean DGAT might be able to preferentially incorporate vernolic acid into TAG. We also demonstrate that the DGAT studied have higher activity when acyl-CoA are fed with *rac*-1,2-DAG than with their corresponding *sn*-1,2-DAG.

EXPERIMENTAL PROCEDURES

Materials. All solvents (HPLC grade or anhydrous), Na, KCl, NaOH, HCl, H_2O_2 (30%), $MgCl_2$, MOPS, DTT, ammonium acetate, glacial acetic acid, Triton X-100, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald®), boiling chips, Whatman No. 1 filter paper, and Whatman TLC plates were purchased from Fisher Scientific (Fair Lawn, NJ). [1-^{14}C]Linoleic acid (55 mCi mmol^{-1}) was purchased from Amersham Biosciences (Piscataway, NJ). [1-^{14}C]Oleoyl CoA (18:1-CoA, 55 mCi mmol^{-1}) supplied in 10 mM sodium acetate (pH 5.2):ethanol (1:1) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). It was diluted about 60-fold with methanol prior to use and stored at –80ºC under argon. 1,2-Dioleoyl-*sn*-glycerol (*sn*-DODAG), 1,2-dioleoyl-*rac*-glycerol (*rac*-DODAG), 1,3-specific lipase (Palatase 20000 L, L4277) from *Rhizomucor miehei*, acyl-

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Abbreviations: 18:1-CoA, oleoyl CoA; DGAT, acyl-CoA:diacylglycerol acyltransferase; *rac*-DODAG, 1,2-dioleoyl-*rac*-glycerol; *sn*-DODAG, 1,2-dioleoyl-*sn*-glycerol; DV, divernolin; *rac*-DVDAG, 1,2-divernoloyl-*rac*-glycerol; *sn*-DVDAG, 1,2-divernoloyl-*sn*-glycerol; MTBE, methyl tertiary butyl ether; MV, monovernolin; *sn*-MVDAG, 1-palmitoyl-2-vernoloyl-*sn*-glycerol; TV, trivernolin; Va-CoA, vernoloyl CoA.

CoA synthetase (A3352), lipase acrylic resin (Novozyme 435, L4777), phospholipase C (P7633), phospholipase A_2 (P0861), 4-Å molecular sieves, antipain, catalase, protease inhibitor cocktail for plant cell and tissue extracts (P9599), diheptadecanoyl PC, ATP, CoASH, ethyl chloroformate, triethylamine, *N*,*N*-4-dimethylaminopyridine, dicyclohexylcarbodiimide, trifluoroacetic anhydride, *sn*-glycero-3-phosphocholine:cadmium chloride adduct (1:1), primulin, Folin & Ciocalteu's phenol reagent (2 N, F9252), and lipid standards other than triacylglyceride (which was from soybean oil [Crisco] purchased from a local grocery store), were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl-2-hydroxy- *sn*-glycero-3-lysophosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Ag 501-X8 resin was purchased from Bio-Rad (Hercules, CA). NH_2 columns (35 mg) were purchased from J.T. Bakers (Phillipsburg, NJ). Sep-Pak tC_{18} cartridges were purchased from Waters (Milford, MA). Silica solid phase extraction columns were purchased from J & W Scientific (Folsom, CA). Liquid scintillation cocktail and 20-mL glass storage vials were purchased from Research Products International Corp. (Mount Prospect, IL). *V. galamensis* oil was purchased from VerTech, Inc. (North Bethesda, MD). Dry hexane and toluene were obtained by adding molecular sieves to HPLC-grade hexane in a 20-mL screw-capped storage vial. Sodium methoxide (4.2%, wt/vol) was made by slowly adding 1.8 g of sodium, which was cut into small pieces when submerged in toluene, into 100 mL methanol under a stream of argon. Diazomethane was synthesized from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide using Mini Diazald® Apparatus according to Aldrich Technical Information Bulletin No. AL-121.

Chemical syntheses. Trivernolin was purified by repeated recrystallization in hexane at –20°C. 1,2-Divernoloyl-*rac*-glycerol (*rac*-DVDAG) was from partial hydrolysis of trivernolin using Palatase (Sigma) and purified by column chromatography. Vernolic (12,13-epoxy-9-octadecenoic) acid was synthesized from saponification of trivernolin with ethanolic NaOH. Briefly, 30 g of trivernolin, 10 g NaOH, and 150 mL of water:ethanol (1:2) were mixed in a 1-L flask and boiled on a hot plate with constant stirring with a magnetic stirrer for 20 min. Ethyl acetate (about 300 mL) was added to the reaction mixture. Subsequently glacial acetic acid was added to neutralize the base to a pH of 5. The ethyl acetate was removed using a separatory, funnel and the water phase was extracted with ethyl acetate two more times. The extracts were combined, washed with water, dried with anhydrous sodium sulfate, and filtered with Whatman No. 1 filter paper. The solvent was evaporated with a rotary evaporator (Buchler Instruments, Fort Lee, NJ). The vernolic acid was purified using column chromatography. Vernolic anhydride was synthesized from vernolic acid using ethyl chloroformate according to Subramanian *et al.* (15) except that methyl tertiary butyl ether (MTBE) was used instead of ether. 1-Palmitoyl-2-vernoloyl-*sn*-glycero-3-phosphocholine and 1,2-divernoloyl-*sn*-glycero-3-phosphocholine were synthesized from vernolic anhydride with 1-palmitoyl-2-hydroxyl-*sn*-glycero-3-phosphocholine and *sn*-glycero-3-phosphocholine:cadmium adduct, respectively, according to Subramanian *et al.* (15) with some modifications. Argon was used for degassing instead of nitrogen. The reaction mixture included both dimethylaminopyridine and dicyclohexylcarbodiimide at a similar molar ratio. 1-Palmitoyl-2-vernoloyl-*sn*-glycerol (*sn*-MVDAG) and 1,2-divernoloyl-*sn*-glycerol (*sn*-DVDAG) were synthesized from their respective PC using phospholipase C according to the manufacturer's activity testing procedure, and the compounds were purified by TLC using the solvent system of hexane/MTBE (60:40, vol/vol). All DAG used for enzyme activity assays were examined via TLC and *sn*-1,3-DAG levels were very low.

[1-^{14}C]Vernolic acid was synthesized by a modified reaction (16) using Novozyme 435 (Sigma). Briefly, [^{14}C]linoleic acid (100 mCi) was dried under a stream of nitrogen and resuspended in 2 mL of toluene. Novozyme 435 (2 mg) and 20 mL of 30% H_2O_2 were added. The reaction mixture was shaken for 4 h at 800 rpm on a VXR orbital shaker (Type VX 7, Janke & Kunkel, Staufen, Germany) at room temperature. A small sample of the reaction mixture was tested on TLC after methylation with diazomethane using a solvent system of hexane/MTBE/acetic acid (85:15:1, by vol) to view the reaction progress. The reaction mixture was kept at –20ºC. This process was repeated until maximum yield was obtained (~40% vernolic acid). The reaction mixture was dried under a stream of nitrogen and methylated with diazomethane. [^{14}C]Methyl vernolate was purified by TLC using a solvent system of hexane/MTBE (88:12, vol/vol). The [^{14}C]vernolic acid methyl ester was saponified with 1 mL of ethanol:2 N NaOH (9:1, vol/vol) at 80°C refluxing under argon for 1 h and extracted with ethyl acetate immediately after acidification by about 0.42 mL 1 N HCl. [^{14}C]Vernoloyl-CoA (Va-CoA) was synthesized using acyl-CoA synthetase (Sigma) according to Taylor *et al.* (17). The Va-CoA was then purified by Sep-Pak tC_{18} cartridges (Waters) according to Gang *et al.* (18) except that the Va-CoA was finally eluted with two volumes of methanol followed by two volumes of butanol. The methanol was evaporated with a stream of nitrogen. The Va-CoA was extracted with butanol and combined with the previous butanol elution. The Va-CoA in butanol was further purified on normal phase silica column using butanol and butanol/water (79:21, vol/vol). The Va-CoA showed a single band on reverse phase TLC using a solvent system of butanol/acetic acid/water (63:10:27, by vol).

Mixed [^{14}C]linoleic acid anhydrides were synthesized from [^{14}C]linoleic acid and trifluoroacetic anhydride in dry hexane at refluxing temperature; the solvent was allowed to leak out of the screw-capped test tube over a period of about 1 h. In detail, [^{14}C]linoleic acid (1 µmol, 55 µCi/µmol) was placed in a 10-mL screw-capped test tube. The solvent was removed under a stream of nitrogen gas and 1 mL of dry hexane was added. To the solution 20 µL trifluoroacetic anhydride was added, and the test tube was flushed with argon and closed. The test tube was placed in boiling water, and the sample was refluxed and hexane was allowed to slowly leave

the test tube over about 1 h. The release of hexane was controlled by slightly loosening the screw cap from the test tube. The sample was resuspended in 1 mL of dry hexane. The yield of [^{14}C]linoleic trifluoroacetic anhydride was checked by TLC using the solvent system hexane/MTBE/acetic acid (85:15:1, by vol). The mixed anhydride ran much higher than linoleic acid on the TLC plate but below linoleic anhydride. If the yield was not satisfactory, the reaction could be repeated until a high yield (over 80%) was obtained.

Vernolic acid is very sensitive to acids, so the method used for mixed linoleic acid anhydride cannot be used for mixed vernolic acid anhydride. Mixed [^{14}C]vernolic anhydride was synthesized by reacting [^{14}C]vernolic acid with an excess of ethyl chloroformate in the presence of triethylamine at room temperature. Briefly, [^{14}C]vernolic acid was dissolved in 1 mL of dry hexane in a 10-mL screw-capped test tube. To the solution 5 μL of 5% triethylamine and 5 μL of 5% ethyl chloroformate were added in dry hexane. The solution was flushed with argon, tightly closed, and shaken at 800 rpm at room temperature for 1.5 h. The reaction mixture was brought to dryness with a stream of nitrogen gas and resuspended in 1 mL dry hexane. The solution was again brought to dryness under a stream of nitrogen gas. The mixed [^{14}C]vernolic anhydride was checked on TLC using a solvent system of hexane/MTBE/acetic acid (85:15:1, by vol).

The procedure for synthesis of [^{14}C]PC was derived from Subramanian *et al.* (15) and Ramstedt and Slotte (19). The labeled mixed FA anhydrides were reacted with an excess of 1-palmitoyl-2-hydroxy-*sn*-glycero-3-lysophosphatidylcholine in the presence of dimethylaminopyridine and dicyclohexylcarbodiimide at room temperature. In detail, the mixed [^{14}C]linoleic acid anhydride or mixed [^{14}C]vernolic anhydride (55 μCi, 1 μmol) synthesized as described above was placed in a 15-mL screw-capped conical test tube and brought to dryness under a stream of nitrogen gas. To the test tube were added 1-palmitoyl-2-hydroxy-*sn*-glycero-3-lysophosphatidylcholine (991 μg, 2 μmol), dimethylaminopyridine (1.22 mg, 10 μmol) and dicyclohexylcarbodiimide (2.06 mg, 10 μmol), all in dry chloroform. The solution was again brought to dryness under a stream of nitrogen gas and resuspended in 40 μL of dry toluene. The sample was flushed with argon and closed tightly with a screw cap. The test tube was shaken for 24 h at 800 rpm at room temperature. The reaction progress was assayed by TLC along with *sn*-1,2-dioleoyl-PC as a standard using solvent systems of chloroform/methanol/water (65:25:4, by vol) and hexane/MTBE (75:25, vol/vol). [^{14}C]PC were synthesized with up to 36% yield for [^{14}C]vernoloyl-PC and 40% for [^{14}C]linoleoyl-PC relative to the labeled mixed FA anhydrides added based on the quantitative analysis using ImageQuant (version 5.2, Amersham Biosciences).

For the analyses of the positional distribution of labeled FA of the [^{14}C]PC, small samples of the labeled PC (0.01 μCi) synthesized as described above were digested with 10 μL phospholipase A_2 (100 units) for 30 min at room temperature in a buffer (100 μL) as described by Egger *et al.* (20) except that the pH was 7.5 instead of 8. The lipids were extracted into chloroform according to Bligh and Dyer (21) and analyzed by TLC using corresponding unlabeled free FA and 1-oleoyl-2-hydroxyl-*sn*-glycero-3-lysophosphatidylcholine as standards. The TLC plates were visualized by phosphorimaging (Amersham). Free FA and lysophosphatidylcholine bands were scraped off the TLC plate and analyzed by a liquid scintillation analyzer (PerkinElmer, Wellesley, MA). The results showed that the digestion went to completion and the percentage of labeled FA located at *sn*-1 and *sn*-2 were 60% and 40% for the palmitoyl-[^{14}C]vernoloyl-PC and 41% and 59% for the palmitoyl-[^{14}C]linoleoyl-PC, respectively. These results indicate that significant acyl exchange between *sn*-1 and *sn*-2 positions occurred during the [^{14}C]PC synthesis reactions.

Microsomal preparations. *V. galamensis* and *S. laevis* were grown in a soil bed outdoors in Lexington, KY. Soybeans (*Glycine max* cv. Jack) were grown in a greenhouse. Microsomes from developing seeds of *V. galamensis*, *S. laevis*, and soybean at a stage of rapid growth and high triglyceride biosynthesis were prepared according to Bafor *et al.* (5), with antipain (5 mg/mL buffer) added according to Lin *et al.* (22). For *V. galamensis* and *S. laevis*, developing seeds used were soft and white after seed coats were removed. Soybean developing seeds used were 9–11 mm in length, and their seed coats were also removed. Protease inhibitor cocktail (33.3 μL g^{-1} of tissue) was added just before tissue homogenation for *V. galamensis* and *S. laevis* but not for soybean since its developing seeds are known to contain large amounts of protease inhibitors that can be released quickly (23). The microsomal pellets were resuspended in a buffer at approximately 1 mL/g tissue. The resuspension buffer was 100 mM potassium phosphate buffer (pH 7.2), 0.1% (wt/vol) of BSA (FA-free), 1,000 units/mL of catalase, and 5 μg/mL of antipain. Aliquots of the microsomal fractions were flash frozen with liquid nitrogen and stored at –80°C. The microsomal PC concentrations were determined in triplicate by GC (Model 5890, Hewlett Packard) using diheptadecanoyl PC as an internal standard according to Christie *et al.* (24). Thirty microliters of microsomal suspension was mixed with 5 μg of diheptadecanoyl PC. The lipids were extracted into chloroform according to Bligh and Dyer (21) and separated on TLC along with 50 μg of soy lecithin as a standard using a solvent system of chloroform/methanol/acetic acid/water (85:17:10:3.5, by vol). The lipid bands were visualized under ultraviolet light after spraying with 0.005% primulin in acetone/water (4:1, vol/vol). The band corresponding to PC was scraped off the plate, and the lipid was methylated with 0.5 mL of sodium methoxide (4.2%, wt/vol) with shaking at 800 rpm for 45 min. One milliliter of hexane was used to extract the FAME, and this was repeated once. The hexane extracts were combined and then washed with 1 mL of 0.9% KCl. The FAME in hexane was brought down to 0.5 mL and analyzed by GC. The area units of the methyl esters of five common FA (palmatic, stearic, oleic, linoleic, and linolenic) were combined for the calculation of microsomal PC levels as compared with those of methyl heptadecanoate. The microsomal

protein concentrations were determined by a modified Lowry method according to Wang (25).

Microsomal assays. Microsomal assay conditions were modified from the DGAT assay as described by Jako *et al.* (26). Microsomal fractions (0.5 nmol PC eq.) were fed with 5 μM [^{14}C]18:1-CoA or [^{14}C]Va-CoA in combinations with *sn*-DODAG, *sn*-MVDAG, *sn*-DVDAG, *rac*-DODAG, *rac*-DVDAG, or no exogenous DAG in a total reaction volume of 200 μL. The *sn*-1,2-DAG were either fed individually or simultaneously. The reaction mixture was in 100 mM potassium phosphate buffer (pH 7.2) containing 1 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM CoASH, and 20% glycerol (vol/vol). The reaction mixture was incubated at 30°C in an incubator with shaking at 100 rpm for 30 min. The microsomal assay reactions were stopped by placing them in ice, which was immediately followed by addition of 1 mL of chloroform/methanol (2:1, vol/vol) containing 50 μg TAG. After vortexing vigorously, 300 μg *V. galamensis* oil was added.

The lipids from microsomal assay reactions were extracted into chloroform according to Bligh and Dyer (21), blown dry, and resuspended in 1 mL ethyl acetate. The ethyl acetate extract was passed through an NH_2 column to removed free FA. Lipids were eluted with a total of 6 mL of ethyl acetate, which was blown down with a stream of nitrogen and loaded onto a Whatman silica gel channel plate with appropriate standards. The TLC was run with hexane/MTBE/acetic acid (75:25:1, by vol) to full length. The nonradioactive compounds were visualized under ultraviolet light after spraying with 0.005% primulin in acetone/water (4:1, vol/vol). Marked radioactive dots were added to the plate to locate the bands precisely. The radioactive bands and dots were visualized using a phosphorimager (Amersham Biosciences), and the bands of interest were scraped off the plates and quantified by a liquid scintillation analyzer (PerkinElmer). The assays had two replicates for each treatment and the experiment was performed three times. Statistical analyses were performed using Statistical Analysis System (SAS Institute, Cary, NC) using GLM procedure with each pair of means analyzed separately, and the significance level was $P < 0.05$.

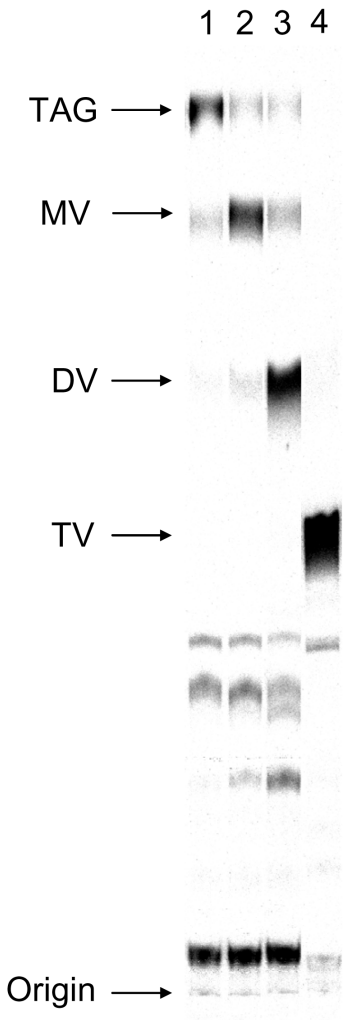

FIG. 1. TLC separation of radiolabeled TAG, monovernolin (MV), divernolin (DV), and trivernolin (TV). The solvent system used was hexane/MTBE/acetic acid (75:25:1, by vol), and the full length was run using a channeled silica gel 60-Å plate. The TLC origin and phosphorimage bands corresponding to the TAG species formed from *V. galamensis* microsomal DGAT assays using fed substrates (lane 1: [^{14}C]oleoyl-CoA and 1,2-dioleoyl-*sn*-glycerol; lane 2: [^{14}C]vernoloyl-CoA and 1,2-dioleoyl-*sn*-glycerol; lane 3: [^{14}C]vernoloyl-CoA and 1-palmitoyl-2-vernoloyl-*sn*-glycerol; lane 4: [^{14}C]vernoloyl-CoA and 1,2-divernoloyl-*rac*-glycerol) are shown. The free FA were removed by passing the sample through an NH_2 column.

RESULTS

To evaluate factors affecting the accumulation of industrially valuable epoxy FA in genetically engineered common oilseed crops, we studied the substrate specificities of DGAT, the enzyme involved in the final synthesis step of TAG, of two natural high accumulators of vernolic acid, *V. galamensis* and *S. laevis*, as compared with soybean, a target crop for genetic engineering for epoxy FA accumulation. Developing seed microsomes were fed with either [^{14}C]18:1-CoA or [^{14}C]Va-CoA in combinations with no exogenous DAG or with DAG. Because vernolic acid is more polar than common FA, the resulting labeled TAG species that have no, one, two, or three vernolic moieties can be cleanly separated on TLC (Fig. 1). Among MAG and DAG species with no, one, or two vernolic acid residues, dioleoylglycerol runs farthest in this separation system but is still significantly behind and easily separated from trivernolin, the slowest-moving TAG species in the present study (data not shown).

For the quantification of microsomes, we determined both microsomal PC and protein concentrations. The ratio between microsomal protein and microsomal PC increased as the seeds developed over time for both *V. galamensis* and *S. laevis* (data not shown). The microsomes used in our studies have microsomal PC levels of 0.28, 0.24, and 0.44 nmol/μL and microsomal protein levels of 2.9, 2.3, and 6.3 μg/μL for *V. galamensis*, *S. laevis*, and soybean, respectively. Microsomal PC quantification was chosen over microsomal protein quantification because DGAT is a membrane-bound enzyme

and might be more correlated with microsomal PC levels. We also verified that the amounts of microsomes and substrates used for the study were within a linear range up to the incubation time of 30 min even for the substrate combinations that had the highest reaction rates. The substrates used contain no, one, or two vernolic residues. The DAG substrates also included stereoisomers of *sn*-1,2-DAG and racemic 1,2(2,3)-DAG, because we found dramatic differences between the two types of DAG in our preliminary DGAT assays. In order to determine whether phospholipid:diacylglycerol acyltransferase was interfering with our microsomal DGAT assays, *Vernonia* microsomes were fed with 5 μM of palmitoyl-[^{14}C]linoleoyl-PC or palmitoyl-[^{14}C]vernoloyl-PC synthesized as described previously instead of [^{14}C]acyl-CoA with no DAG, 100 μM *sn*-DODAG, or 100 μM *sn*-DVDAG using otherwise the same DGAT assay as described previously. The results showed no detectable TAG formed (data not shown).

The microsomal DGAT assay results for *V. galamensis* are presented in Figure 2. When [^{14}C]acyl-CoA and *sn*-1,2-DAG were fed individually to developing seed microsomes, the most preferred substrate combination for *V. galamensis* DGAT was Va-CoA with *sn*-DVDAG, followed by Va-CoA with *sn*-MVDAG, whereas the lowest DGAT activities were found with the substrate combination of Va-CoA with *sn*-DODAG, followed by 18:1-CoA with *sn*-DODAG (Fig. 2A). With either *sn*-MVDAG or *sn*-DVDAG, Va-CoA was preferred over 18:1-CoA. With either 18:1-CoA or Va-CoA, *sn*-DVDAG was preferred over *sn*-MVDAG. When the three *sn*-1,2-DAG were fed simultaneously with either [^{14}C]18:1-CoA or [^{14}C]Va-CoA for competitive DGAT assays, the results were similar to those when DAG were fed individually except that the higher DGAT activities toward the substrate combination of Va-CoA with *sn*-DVDAG were more dramatic (Fig. 2B). This is understandable because the substrate combination of Va-CoA with *sn*-DVDAG was dominant in the competition for the labeled Va-CoA. When Va-CoA but no exogenous DAG were fed to the samples, the DGAT activities using endogenous common FA DAG, MVDAG, and DVDAG also increased in this order although the overall DGAT activities were much lower, probably due to lower amounts of the endogenous microsomal DAG available compared with the samples fed *sn*-1,2-DAG (Fig. 2C). When 18:1-CoA but no exogenous DAG were fed, the DGAT activities using endogenous common FA DAG were higher than

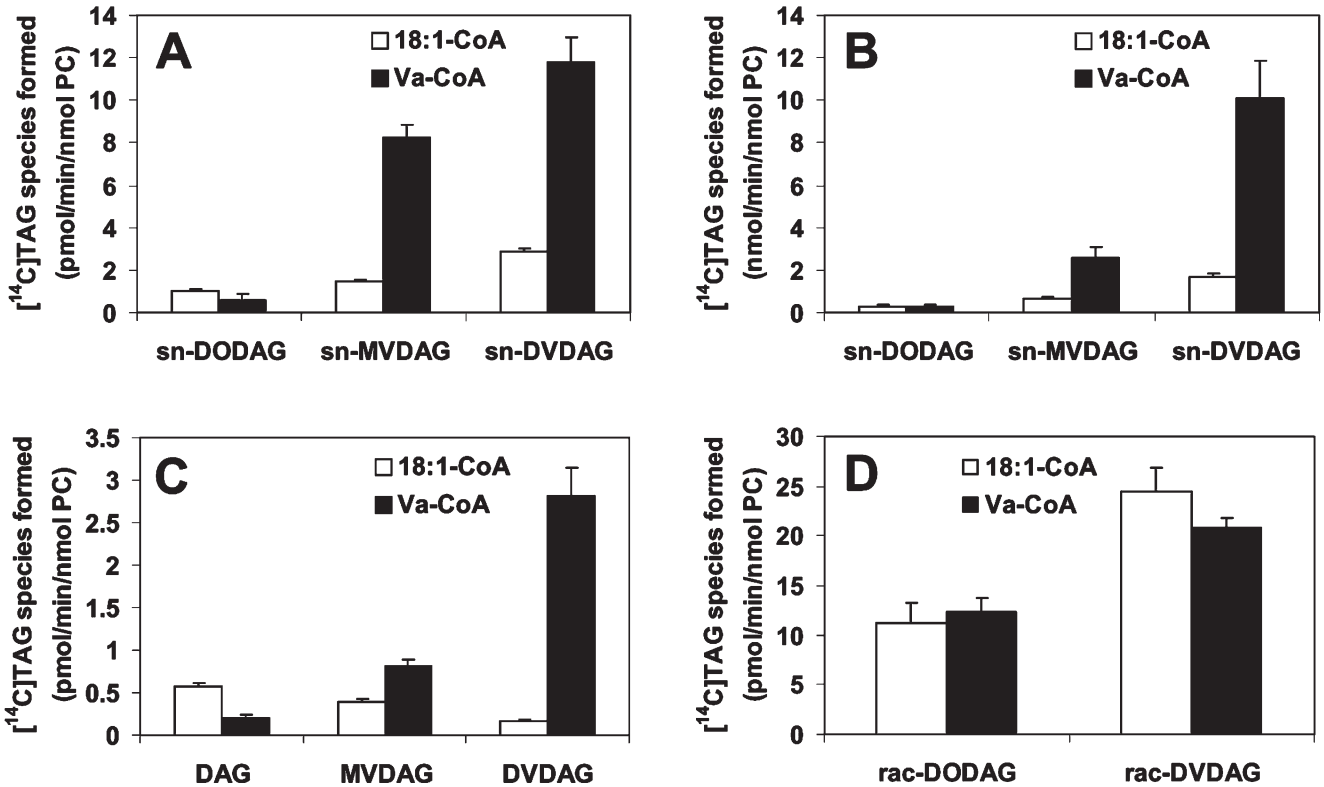

FIG 2. TAG formed from *V. galamensis* microsomal DGAT assays. The V. galamensis microsomes (eq. of 0.5 nmol microsomal PC) from developing seeds were fed with either [^{14}C]oleoyl-CoA (18:1-CoA) or [^{14}C]vernoloyl-CoA (Va-CoA) in combinations with or without exogenous DAG at 100 mM. The DAG treatments include exogenous 1,2-dioleoyl-*sn*-glycerol (*sn*-DODAG), 1-palmitoyl-2-vernoloyl-*sn*-glycerol (*sn*-MVDAG), or 1,2-divernoloyl-*sn*-glycerol (*sn*-DVDAG) added separately (A) or together (B); no exogenous DAG added (C); and exogenous 1,2-dioleoyl-*rac*-glycerol (*rac*-DODAG) or 1,2-divernoloyl-*rac*-glycerol (*rac*-DVDAG) added separately (D). The reaction mixture (200 μL) also included 1 mM $MgCl_2$, 0.5 mM CoASH, and 0.5 mM ATP and was incubated for 30 min at 30°C with shaking at 100 rpm. Values are mean ± SD (n = 6) of three experiments each with two replications.

those using MVDAG, which were in turn higher than those using DVDAG. These results were probably because the microsomal common FA DAG, MVDAG, and DVDAG were presumably in a steep decreasing order. When *rac*-1,2-DAG were fed individually with either [^{14}C]18:1-CoA or [^{14}C]Va-CoA, DGAT activities were much higher than with corresponding *sn*-1,2-DAG (Fig. 2D). With 18:1-CoA and Va-CoA, respectively, *rac*-DODAG was incorporated into TAG at about 10- and 20-fold higher levels relative to *sn*-DODAG, whereas *rac*-DVDAG was favored by about 8- and 2-fold more than *sn*-DVDAG. With either 18:1-CoA or Va-CoA, *rac*-DVDAG was preferred over *rac*-DODAG (about 2-fold). With *rac*-DODAG, no preference was found between 18:1-CoA and Va-CoA. However, with *rac*-DVDAG, 18:1-CoA was slightly preferred over Va-CoA ($P < 0.05$). DGAT substrate preference for unusual FA in the forms of acyl-CoA and/or DAG has been reported by several studies (12,14,27), most notably for medium-chain FA in two *Cuphea* species and a hydroxy FA in castor. However, to the best of our knowledge the dramatic difference in DGAT activities for *sn*-1,2-DAG and *rac*-1,2-DAG have not been reported. The lack of differences among *sn*-1,2-DODAG, *sn*-2,3-DODAG, and *rac*-1,2-DODAG found by other researchers (14,28) might have been due to different plant species under investigation.

The microsomal DGAT assay results for *S. laevis* are presented in Figure 3. The overall substrate preference pattern was quite similar to the results from *V. galamensis*. The DGAT activities for *S. laevis* were generally lower than those for *V. galamensis*. When [^{14}C]acyl-CoAs and *sn*-1,2-DAG were fed individually, the most preferred substrate combination for *S. laevis* DGAT was also Va-CoA with *sn*-DVDAG followed by Va-CoA with *sn*-MVDAG, whereas the lowest DGAT activities were found with the substrate combination of Va-CoA with *sn*-DODAG (Fig. 3A). Similar to the results from *V. galamensis*, with either *sn*-MVDAG or *sn*-DVDAG, Va-CoA was preferred over 18:1-CoA, and with either 18:1-CoA or Va-CoA, *sn*-DVDAG was preferred over *sn*-MVDAG. However, with 18:1-CoA, no difference was found between *sn*-DODAG and *sn*-MVDAG. When the three *sn*-1,2-DAG were fed simultaneously with either [^{14}C]18:1-CoA or [^{14}C]Va-CoA, the results were similar to those fed individually (Fig. 3B). With 18:1-CoA, *sn*-MVDAG was slightly preferred over *sn*-DODAG, and this is different from the results when the DAG were fed individually. This small difference might be due to the direct substrate competition involved when the DAG were fed simultaneously. When Va-CoA but no exogenous DAG were fed to the samples, the DGAT activities using endogenous common FA DAG, MVDAG, and

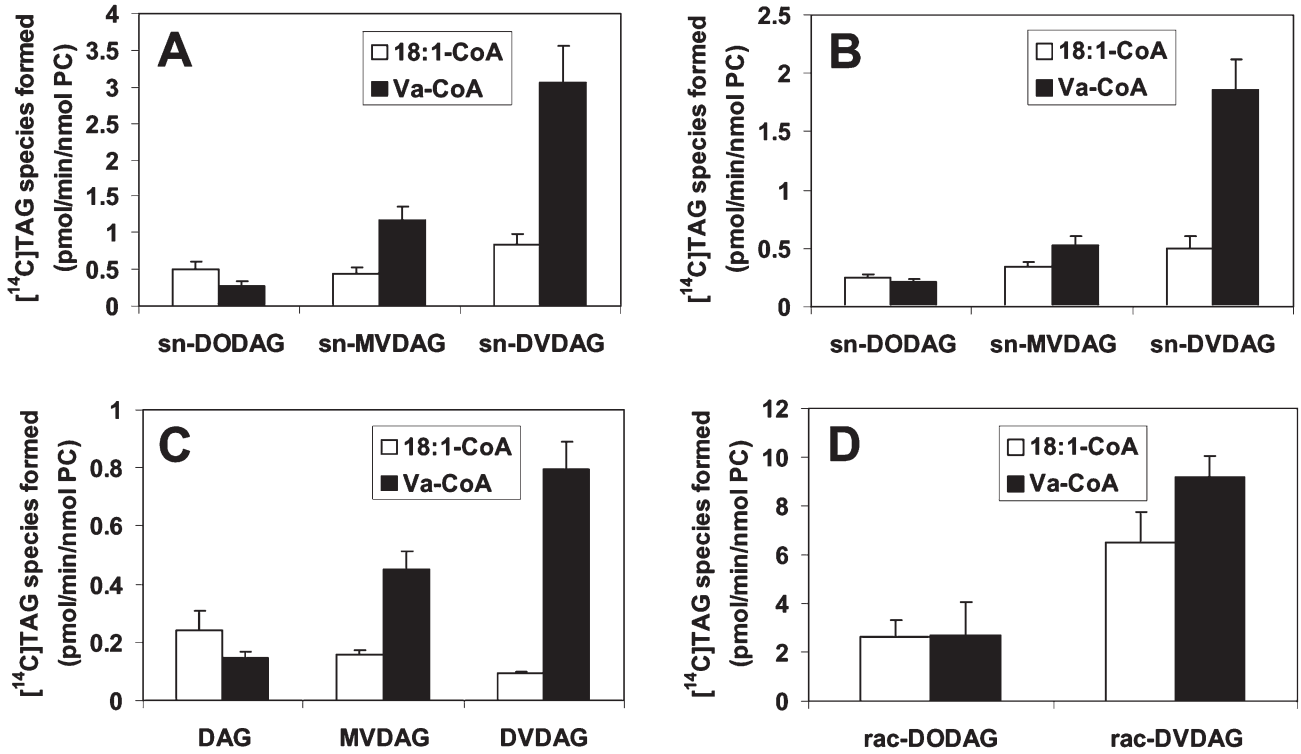

FIG 3. TAG formed from *S. laevis* microsomal DGAT assays. The *S. laevis* microsomes (eq. of 0.5 nmol microsomal PC) from developing seeds were fed with either [^{14}C]oleoyl-CoA (18:1-CoA) or [^{14}C]vernoloyl-CoA (Va-CoA) in combinations with or without exogenous DAG at 100 mM. The DAG treatments include exogenous 1,2-dioleoyl-*sn*-glycerol (*sn*-DODAG), 1-palmitoyl-2-vernoloyl-*sn*-glycerol (*sn*-MVDAG), or 1,2-divernoloyl-*sn*-glycerol (*sn*-DVDAG) added separately (A) or together (B); no exogenous DAG added (C); and exogenous 1,2-dioleoyl-*rac*-glycerol (*rac*-DODAG) or 1,2-divernoloyl-*rac*-glycerol (*rac*-DVDAG) added separately (D). The reaction mixture (200 µL) also included 1 mM $MgCl_2$, 0.5 mM CoASH, and 0.5 mM ATP and was incubated for 30 min at 30°C with shaking at 100 rpm. Values are mean ± SD (n = 6) of three experiments each with two replications.

DVDAG were of the same pattern as described for *V. galamensis* but with lower overall activities (Fig. 3C). When *rac*-1,2-DAG were fed individually with [^{14}C]acyl-CoAs, DGAT activities were much higher than with corresponding *sn*-1,2-DAG (Fig. 3D). With 18:1-CoA and Va-CoA respectively, *rac*-DODAG was about 5- and 10-fold more incorporated into TAG than *sn*-DODAG, whereas *rac*-DVDAG was about 8- and 3-fold higher than *sn*-DVDAG. With either 18:1-CoA or Va-CoA, *rac*-DVDAG was preferred over *rac*-DODAG (about 2.5-fold). With *rac*-DODAG, no preference was found between 18:1-CoA and Va-CoA. However, with *rac*-DVDAG, Va-CoA was slightly preferred over 18:1-CoA ($P < 0.05$), which is different from the results from *V. galamensis*.

The microsomal DGAT assay results for soybean are presented in Figure 4. When [^{14}C]acyl-CoA and *sn*-1,2-DAG were fed individually, DGAT activities with 18:1-CoA were 4- to 5-fold higher than with Va-CoA (Fig. 4A). With 18:1-CoA, *sn*-DODAG was slightly preferred over *sn*-MVDAG, which in turn was preferred over *sn*-DVDAG. However, with Va-CoA, no difference in DGAT activity was found for the three *sn*-1,2-DAG. When the three *sn*-1,2-DAG were fed simultaneously with either [^{14}C]18:1-CoA or [^{14}C]Va-CoA for competitive DGAT assays, the results were quite similar to those fed individually in DGAT substrate preferences but lower in overall DGAT activities (Fig. 4B). DGAT activities with 18:1-CoA were 3- to 4-fold higher than those with Va-CoA. With 18:1-CoA, *sn*-DODAG was slightly preferred over *sn*-MVDAG and *sn*-DVDAG, which had no difference between them. With Va-CoA, no difference in DGAT activity was found for the *sn*-DODAG and *sn*-DVDAG both of which were slightly higher than *sn*-MVDAG. When Va-CoA but no exogenous DAG were fed to the samples, the DGAT activities with 18:1-CoA was about 5-fold higher than those with Va-CoA, and no TAG formed from endogenous MVDAG and DVDAG (which are not present) was found (Fig. 4C). When *rac*-1,2-DAG were fed individually with [^{14}C]acyl-CoAs, DGAT activities were much higher than with corresponding *sn*-1,2-DAG (Fig. 4D). With 18:1-CoA and Va-CoA, respectively, *rac*-DODAG was about 4- and 5-fold more incorporated into TAG than *sn*-DODAG, whereas *rac*-DVDAG was about 6- and 10-fold higher than *sn*-DVDAG. With either *rac*-DODAG or *rac*-DVDAG, DGAT activities with 18:1-CoA were 2- to 3-fold higher than those with Va-CoA. With 18:1-CoA, no preference was found between *rac*-DODAG and *rac*-DVDAG. However, with Va-CoA, *rac*-DVDAG was slightly preferred over *rac*-DODAG ($P < 0.05$).

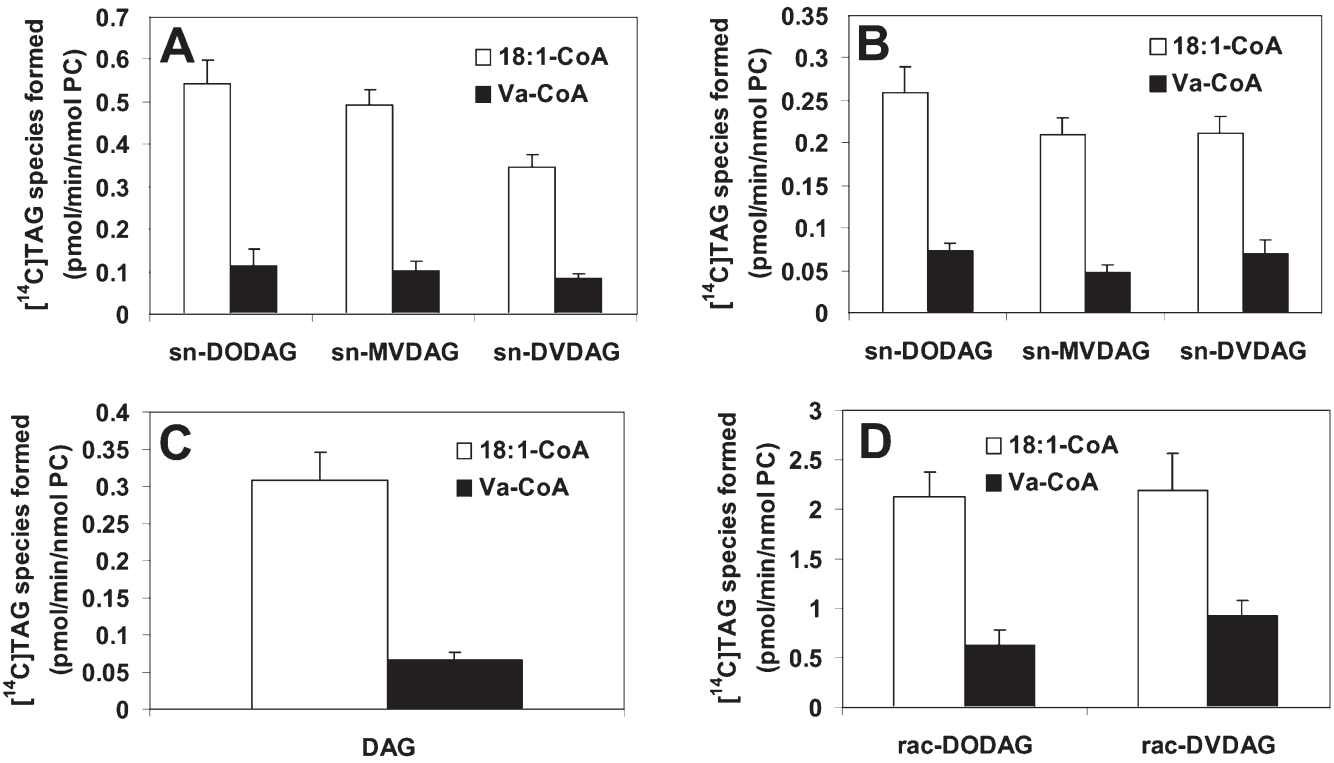

FIG 4. TAG formed from soybean microsomal DGAT assays. The soybean microsomes (eq. of 0.5 nmol microsomal PC) from developing seeds were fed with either [^{14}C]oleoyl-CoA (18:1-CoA) or [^{14}C]vernoloyl-CoA (Va-CoA) in combinations with or without exogenous DAG at 100 mM. The DAG treatments include exogenous 1,2-dioleoyl-*sn*-glycerol (*sn*-DODAG), 1-palmitoyl-2-vernoloyl-*sn*-glycerol (*sn*-MVDAG), or 1,2-divernoloyl-*sn*-glycerol (*sn*-DVDAG) added separately (A) or together (B); no exogenous DAG added (C); and exogenous 1,2-dioleoyl-*rac*-glycerol (*rac*-DODAG) or 1,2-divernoloyl-*rac*-glycerol (*rac*-DVDAG) added separately (D). The reaction mixture (200 µL) also included 1 mM $MgCl_2$, 0.5 mM CoASH, and 0.5 mM ATP and was incubated for 30 min at 30°C with shaking at 100 rpm. Values are mean ± SD (n = 6) of three experiments each with two replications.

DISCUSSION

The identification of epoxygenases from high vernolic acid accumulators has opened up an opportunity for genetically engineering oilseed crops such as soybean to produce vernolic acid (6,7), an important industrial feedstock for a variety of applications. However, genetically transformed arabidopsis and soybeans with epoxygenase alone can only result in a low accumulation of vernolic acid (7,9,10,29). In the present study, we found that the enzymes involved in the final step of synthesis of TAG, DGAT in both natural high accumulators of vernolic acid, *V. galamensis* and *S. laevis*, are highly selective for substrates with vernolic moieties in a manner consistent with their accumulation in their seed oils, with the most favorable substrate combination being Va-CoA and *sn*-DVDAG. The DGAT of the common oilseed crop soybean shows relatively lower activity for substrates with vernolic moieties. Instead, soybean DGAT has higher activity with 18:1-CoA than Va-CoA, and slightly higher activity with *sn*-DODAG compared with *sn*-1,2-DAG with vernolic moieties when 18:1-CoA was the cosubstrate. Lin *et al.* (30,31) investigated the incorporation of oleate and ricinoleate into microsomes from soybean and castor, a high accumulator of a hydroxy FA. Similar to our results, they found that castor selectively incorporated ricinoleate into TAG. However, they reported that soybean did not distinguish between ricinoleic acid and oleate. The lack of selectivity in soybeans might be due to the different unusual FA under investigation, or due to the significantly different experimental conditions employed in their study. They also suggested that the removal of ricinoleate and oleate from PC was different because of differing specificities of phospholipase A_2 that might affect the incorporation of the FA into TAG.

Surprisingly, the *rac*-1,2-DAG tested were all much better substrates than their corresponding *sn*-1,2-DAG for the DGAT of all three species, *V. galamensis*, *S. laevis*, and soybean. When *rac*-1,2-DAG were used in the studies, the strong selectivity of *V. galamensis* and *S. laevis* DGAT for substrates with vernolic moieties as described above was diminished. These results might be due to dramatically higher DGAT activities with *sn*-2,3-DAG possibly because of the lack of regulation by the DGAT from the plant species for *sn*-2,3-DAG, which do not occur to any extent in seeds. Such results might also be due to significant synergetic effects between *sn*-1,2-DAG and *sn*-2,3-DAG for DGAT activity. DGAT use both acyl-CoA and DAG as substrates; the important point might not be so much the relative activities between *sn*-1,2-DAG and *sn*-2,3-DAG but the change in selectivity toward acyl-CoA. Further investigations, such as the direct comparison of DGAT activities using *sn*-1,2-DAG and *sn*-2,3-DAG, would be useful to determine the basis for this. Previous studies comparing *sn*-1,2-DODAG, *sn*-2,3-DODAG, and *rac*-1,2-DODAG in safflower (*Carthamus tinctorius*) (28) and comparing *sn*-1,2-DODAG with *rac*-1,2-DODAG in *Cuphea procumbens*, sunflower (*Helianthus annuus*), and castor (14) showed no differences in terms of DGAT activities. This is very different from our results, in which all three plant species tested for DGAT activities with both pairs of DAG, *sn*-DODAG/*rac*-DODAG and *sn*-DVDAG/*rac*-DVDAG showed dramatically different activities. The differences between our results and the prior studies might be due to the different species under investigation. Further, previous researchers tested only dioleins with those species. Our results suggest that it may be worthwhile to use *sn*-1,2-DAG for DGAT assays even though it may be more difficult to prepare *sn*-1,2-DAG with unusual FA moieties.

Several studies have shown that the DGAT of unusual FA accumulators have substrate preferences for the unusual FA CoAs and/or unusual FA DAG, most notably in castor and several *Cuphea* species (12–14,27). In *C. carthagenensis*, the DGAT preference of lauroyl CoA over 18:1-CoA with *sn*-DODAG was not high, and the selectivity might be partly due to the higher solubility of lauroyl CoA than 18:1-CoA because the selectivity seemed to be correlated with the microsomal assay temperatures (27). In *C. lanceolata*, with 18:1-CoA, the DGAT preference for 1,2-dicaproyl-*sn*-glycerol over *sn*-DODAG was about 3-fold (12). In *C. procumbens*, the DGAT preference for caproyl CoA over 18:1-CoA with both 1,2-dicaproyl-*rac*-glycerol and *rac*-DODAG was about 2- to 3-fold (14). However, the DGAT preference for 1,2-dicaproyl-*rac*-glycerol over *rac*-DODAG was over 10-fold. They also studied castor DGAT and found that the DGAT was 2- to 4-fold more active with ricinoleoyl-CoA than with 18:1-CoA either with *rac*-DODAG or 1,2-diricinoleoyl-*rac*-glycerol (14). With either acyl-CoA, 1,2-diricinoleoyl-*rac*-glycerol was about 2-fold more incorporated into TAG than *rac*-DODAG by castor DGAT. Another study with castor using 18:1-CoA and *sn*-1,2-DAG showed that 1,2-diricinoloyl-*sn*-glycerol was incorporated into TAG by DGAT about 3-fold higher than *sn*-DODAG (12). Interestingly, monoricinoleoyl DAG was reported to have significantly lower incorporation into TAG than diricinoleoyl DAG. In a more recently study by He *et al.* (13), a castor DGAT was expressed in yeast and its substrate specificities examined. With 18:1-CoA, the castor DGAT expressed in yeast was about 2-fold more active with 1,2-diricinoleoyl-*rac*-glycerol than with diolein and dipalmitolein, although it was not reported whether the common FA DAG were racemic or of certain stereospecificity. Castor DGAT might be expected to be similar to *V. galamensis* and *S. laevis* DGAT because they are all natural high accumulators of oxygenated FA synthesized by FAD2-like enzymes from PC and may use similar mechanisms for the high accumulation of the unusual FA (6,9,32,33). In comparison, our results have more clearly demonstrated the high selectivity of *V. galamensis* and *S. laevis* DGAT for vernolic acid incorporation into TAG due to the discovery of the dramatic difference in activities of the tested DGAT with *rac*-1,2-DAG and *sn*-1,2-DAG. *V. galamensis* DGAT also showed slightly higher substrate selectivity for substrates with vernolic moieties than *S. laevis* DGAT.

Vernolic acid is known to be synthesized from linoleic acid at the *sn*-2 position of PC by an epoxygenase (5). The mechanisms for the channeling or other means of selective accumu-

lation of epoxy FA such as vernolic acid into TAG are largely unknown. Phospholipid:diacylglycerol acyltransferase and phospholipase have been shown to play major roles in removing vernolic acid from phospholipids in *Crepis palaestina* and *Euphorbia lagascae* (34). They did not show the absolute rates of the reactions, so it is difficult to compare the phospholipid:diacylglycerol acyltransferases with DGAT in terms of their relative importance for TAG synthesis. In the present study, we could not detect any phospholipid:diacylglycerol acyltransferase activities in *V. galamensis* microsomes by replacing [^{14}C]acyl-CoA with [^{14}C]PC under otherwise identical conditions for our DGAT assays with or without exogenous *sn*-1,2-DAG. This may be due to suboptimal assay conditions for phospholipid:diacylglycerol acyltransferase or due to intrinsically very low activities for the enzyme. Although it is not certain how relevant the microsomal DGAT activity is to accumulation of TAG *in vivo*, our results indicate that DGAT might play an important role in the selective accumulation of vernolic acid in *V. galamensis* and *S. laevis*. Verification by cloning and functional analyses of the genes encoding these enzymes will be very helpful to gain further insights and might yield a *V. galamensis* DGAT gene that would be useful, along with epoxygenases, for the high-level production of vernolic acid in genetically engineered common oil crops.

In summary, we have found that *V. galamensis* and *S. laevis* DGAT can preferentially incorporate substrates with vernolic moieties into TAG, whereas soybean DGAT does not have such specificities. In combination with acyl-CoA, *rac*-1,2-DAG are much better substrates than their corresponding *sn*-1,2-DAG for *V. galamensis*, *S. laevis*, and soybean DGAT. These studies suggest that DGAT might be an important enzyme for selective incorporation of vernolic acid into TAG in *V. galamensis* and *S. laevis*.

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Impact of 20:4n-6 Supplementation on the Fatty Acid Composition and Hemocyte Parameters of the Pacific Oyster *Crassostrea gigas*

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ABSTRACT: Arachidonic acid (20:4n-6, ArA) and its eicosanoid metabolites have been demonstrated to be implicated in immune functions of vertebrates, fish, and insects. Thus, the aim of this study was to assess the impact of ArA supplementation on the FA composition and hemocyte parameters of oysters *Crassostrea gigas*. Oyster dietary conditioning consisted of direct addition of ArA solutions at a dose of 0, 0.25, or 0.41 μg ArA per mL of seawater into tanks in the presence or absence of T-*Iso* algae. Results showed significant incorporation of ArA into gill polar lipids when administered with algae (up to 19.7%) or without algae (up to 12.1%). ArA supplementation led to an increase in hemocyte numbers, phagocytosis, and production of reactive oxygen species by hemocytes from ArA-supplemented oysters. Moreover, the inhibitory effect of *Vibrio aestuarianus* extracellular products on the adhesive proprieties of hemocytes was lessened in oysters fed ArA-supplemented T-*Iso*. All changes in oyster hemocyte parameters reported in the present study suggest that ArA and/or eicosanoid metabolites affect oyster hemocyte functions.

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The dietary impact of long-chain n-3 and n-6 essential PUFA has been extensively studied in human medical research, including the modulation of the human immune system. Thus, long-chain n-3 PUFA have been shown to have anti-inflammatory and immunomodulatory properties for a number of immune functions including phagocytosis, reactive oxygen species production, and lymphocyte proliferation (1,2). The impact of dietary long-chain n-6 PUFA on immunity however has been less well investigated and is still controversial, with both proinflammatory and immunosuppressive properties having been associated with these molecules (2). *In vitro* studies have focused on the mechanisms by which n-6 PUFA, especially arachidonic acid (20:4n-6, ArA) and its eicosanoid metabolites, can affect immune functions (3–8). These studies demonstrated that ArA, generally found in high amounts in membranes of higher vertebrates (human, rat), can influence phagocytosis, NADPH oxidase activation, actin polymerization, and Ca^{2+} release, likely via its eicosanoid metabolites.

Similar studies on fish nutrition have evaluated the impact of n-3 and n-6 dietary PUFA on immune functions (9–15). Thus, Lin and Shiau (13) demonstrated that lipid-supplemented diets resulted in increased numbers of white blood cells, enhanced phagocytosis, and the generation of reactive oxygen species in grouper *Epinephelus malabarius* when compared with fish fed lipid-poor diets. Wu *et al.* (15) investigated more specifically the effect of long-chain n-3 PUFA (20:5n-3 [EPA] and 22:6n-3 [DHA]) on the immune functions of the grouper *E. malabarius*. The authors reported that DHA enhanced phagocytic function and T-cell proliferation and was superior to EPA in influencing the cellular defense responses of the grouper. Moreover, eicosanoid metabolites derived from ArA PUFA are also significant in fish immunity. Tafalla *et al.* (16) demonstrated that LTB_4 in leukocyte-derived supernatants have an antiviral activity against viral hemorrhagic septicemia virus in the turbot *Scophthalmus maximus*. However, the authors did not exclude the involvement of other factors such as PGE_2.

In bivalves, for more than two decades, studies have focused on the determination of the nutritional values of various algal species to support and improve larval development, growth, and metamorphosis under hatchery conditions (17–23). Fewer studies have investigated the impact of nutrition on immune responses of bivalves (24–26). Recently, Delaporte *et al.* (26) have demonstrated that feeding Pacific oysters *Crassostrea gigas* and Manila clams *Ruditapes philippinarum* with cultures of the microalga *Chaetoceros calcitrans* (a diatom) led to increases in hemocyte numbers, phagocytosis, and reactive oxygen species production. These authors suggested that the FA composition of *Chaetoceros calcitrans*, characterized by high proportions of EPA (17.8%) and ArA (2.0%), may be responsible for the changes observed in immune responses of both species, as demonstrated for vertebrates and fishes fed on different oils. Similarly, Hégaret *et*

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Abbreviations: AASH, anti-aggregant solution for hemocytes; ArA, arachidonic acid; AU, arbitrary units; DCFH-DA, 2′7′-dichlorofluorescein diacetate; DPI, diphenylene iodonium chloride; DW, dry weight; ECP, extracellular products; FSSW, filtered sterile seawater; ROS, reactive oxygen species; WW, wet weight.

al. (24) showed that immune parameters of the Eastern oyster *Crassostrea virginica* were modulated by the quality of the algal diet. Nonetheless, no specific PUFA could be clearly identified as responsible for those changes. Lastly, dietary conditioning using lipid emulsion was used to assess the impact of EPA on hemocyte parameters of the oyster *C. gigas* (25). In this study, a decrease of phagocytosis and reactive oxygen species production was temporarily observed for oysters fed the highest dose of EPA. These results suggest that this PUFA may have an impact on hemocyte function, but unfortunately without excluding a concomitant impact of ArA because the lipid emulsion also contained a significant amount of ArA.

The aim of this current study was to specifically determine the impact of dietary ArA on hemocyte parameters of *C. gigas*. To do this, a new supplementation method developed by Séguineau *et al.* (27) based on the direct addition of individual PUFA along with T-*Iso* algae (deficient in ArA) was used.

EXPERIMENTAL PROCEDURES

Oyster dietary treatments. One-year-old oysters, *C. gigas* (Thunberg 1793), produced in 2003 at the IFREMER hatchery in La Tremblade (Charente, France), were conditioned in May 2004 at the IFREMER shellfish laboratory in Argenton (Finistère, France) in 50-L tanks. Oysters were acclimated to the experimental temperature of 17°C for 2 wk prior to the dietary experiment. After the acclimation period, oysters were divided randomly and distributed into 9 tanks (70 oysters per tanks, 3 replicates per treatment) filled with 45 L of 20-µm-filtered seawater, mixed and aerated using an immersed pump. Thereafter, oysters were fed *Isochrysis* sp., clone T-*Iso*, supplemented with ArA solution for 4 wk. T-*Iso* was chosen for this experiment because of its low (0.1%) concentration of ArA (26). The daily algal ration was established at 4% algal dry weight per oyster dry weight. A peristaltic pump was adjusted to distribute the algae ration over an 18-h period per day. ArA (Sigma A-9376) was dissolved in ethanol at the concentration of 10 mg mL^{-1} according to the procedure of Séguineau *et al.* (27). This ArA solution was further diluted in ethanol to allow addition of an equal volume of ethanol per tank and to obtain the following final concentrations: 0 µg (control ethanol solution), 0.25 µg, or 0.41 µg of ArA per mL of sea water. ArA solutions were added twice a day (half dose in the morning and half dose in the evening in order to avoid ArA oxidation). Tanks and oysters were cleaned daily. Every 2 wk, 15 oysters were sampled for biochemical analyses and 20 oysters for immune analyses. After the first sampling, volume of seawater per tank was adjusted in order to maintain a stable effective volume per oyster during the experiment. No mortalities were recorded over the whole experiment.

One month later, a separate control experiment was conducted for a period of 2 wk with the same experimental protocol to assess the assimilation of dissolved ArA without algal supply in oysters from the same origin.

Condition index and biochemical composition. At each sampling date, whole oyster, shell, and wet flesh weights were measured on 15 oysters. The condition index of oysters was calculated, as described by Walne and Mann (28), following the formula (dry flesh weight)/(dry shell weight) × 1,000. Thereafter, three pools constituted by the wet flesh tissues of five animals were generated. Pools were frozen in liquid nitrogen (–196°C) and treated as described in Delaporte *et al.* (29). Total lipid content was estimated according to Bligh and Dyer (30), and carbohydrate and protein contents were measured colorimetrically following the procedures of Dubois *et al.* (31) and Lowry *et al.* (32), respectively. Results are expressed as mg of carbohydrate, lipid, or protein per mg of oyster dry flesh weight.

FA analysis of oyster gills: Sampling and extraction. Gills were dissected from the same animals utilized for the hemocyte-parameter analyses. Three pools of gills from five animals were constituted and frozen in liquid nitrogen at –196°C. Pooled gill samples were ground with a Dangoumeau homogenizer, and 300 mg of the ground tissue were transferred to a tube containing 6 mL of chloroform-methanol mixture (2:1, vol/vol). After centrifugation, the lipid extract was transferred to a clean tube, sealed under nitrogen, and stored at –20°C.

Separation of polar and neutral lipids. Neutral and polar lipids of gill lipid extracts were purified on a silica gel microcolumn according to Marty *et al.* (33) and analyzed as described in Delaporte *et al.* (29). Briefly, an aliquot of the lipid extract in chloroform/methanol was evaporated to dryness. Then, lipids were redissolved in a chloroform-methanol mixture (98:2, vol/vol) and placed on a silica gel microcolumn (30 × 5 mm i.d. Kieselgel, 70-230 mesh [Merck] previously heated to 450°C and deactivated with 5 wt % water). Neutral lipids were eluted with a chloroform-methanol mixture (98:2, vol/vol), and the polar lipids were eluted separately with methanol. After transesterification of the neutral and polar lipid extracts from gills by 10% wt/wt boron trifluoride/methanol (34), FAME were analyzed in a gas chromatograph equipped with an on-column injector, a DB-Wax (30 m × 0.25 mm, 0.25 µm film thickness) capillary column, and a flame ionization detector. Hydrogen was used as the carrier gas. The FA were identified by comparing their retention times with those of standards and confirmed by GC–MS.

FA composition of gills was expressed as weight percentage of the total FA of each lipid fraction. Total FA content (neutral + polar lipids) per gill sample was expressed as µg of FA per mg of gill wet weight.

As polar lipids generally provide a good approximation of cell membrane lipids and are the dominant lipids in hemocyte and gill tissues (26,29,35), only FA composition values of the polar lipid fraction of gill lipid extracts were presented in this study to simplify the presentation of FA data.

Measurements of immunological parameters by flow cytometry. Measurements of hemocyte types, numbers, and functions were performed on a FACScalibur flow cytometer (B-D Biosciences, San Jose, CA) equipped with a 488-nm

argon laser. Hemolymph sampling and hemocyte parameter measurements are described next. For each assay, samples were filtered through an 80-μm mesh prior to flow cytometer analysis in order to eliminate large debris (>80 μm).

Hemolymph sampling. Hemolymph was withdrawn from individual oysters using a 1-mL plastic syringe fitted with a 25-gauge needle via a notch adjacent to the adductor muscle created just prior to the bleeding. All hemolymph samples were examined microscopically for contamination (e.g., oocytes, sperm, algae) and stored individually in microtubes at 0°C. Three pools of five individual samples were used for the following immunological assays.

Hemocyte viability, total hemocyte, and subpopulation concentrations. An aliquot of 100 μL of pooled hemolymph was transferred into a tube containing a mixture of 200 μL anti-aggregant solution for hemocytes (AASH) and 100 μL filtered sterile seawater (FSSW). AASH was prepared according to Auffret and Oubella (36). Hemocyte DNA was stained with two fluorescent DNA/RNA specific dyes, SYBR Green I (final concentration 10×) and propidium iodide (PI, final concentration 20 μg mL^{-1}), in darkness at room temperature for 60 min before flow-cytometric analysis. SYBR Green I permeates both dead and live cells, whereas PI permeates only through membranes of dead cells. SYBR Green fluorescence was measured at 500–530 nm (green) by flow cytometry whereas PI fluorescence was detected at 550–600 nm (red). Thus, by counting the cells stained by PI and cells stained by SYBR Green, it was possible to estimate the percentage of viable cells in each sample.

All SYBR Green I stained cells were plotted on a Forward Scatter (FSC, size) and Side Scatter (SSC, granularity) cytogram allowing identification of hemocyte subpopulations. Three subpopulations were distinguished according to their size and granularity and termed granulocytes (high FSC and high SSC), hyalinocytes (high FSC and low SSC) and small agranulocytes (low FSC and low SSC). Total and differential hemocyte concentrations are expressed as number of cells per mL.

Phagocytosis assay. An aliquot of 100 μL pooled hemolymph, diluted with 100 μL of FSSW, was mixed with 30 μL of YG 2.0 μm fluoresbrite microspheres, diluted to 2% in FSSW (Polysciences, Eppelheim, Germany). After 120 min of incubation at 18°C, hemocytes were fixed with 230 μL of a 6% formalin solution and analyzed at 500–530 nm by flow cytometry to detect cells containing fluorescent beads. The percentage of phagocytic cells was estimated by the percentage of hemocytes that had engulfed three beads and more according to Delaporte *et al.* (26).

Adhesion capacity. The adhesive capacity of *C. gigas* hemocytes was assessed by modifying the procedure of Choquet *et al.* (37). In the present study, live pathogenic bacteria were replaced by the extracellular products (ECP) of the *Vibrio aestuarianus*, previously demonstrated to inhibit adhesive capacity of *C. gigas* hemocytes (38). Two subsamples of each hemolymph pool were distributed (100 μL per well) in 24-well microplates. An aliquot of 100 μL FSSW was added to the first subsample as a control, and 100 μL ECP suspension was added to the second (30 μg protein mL^{-1} final concentration). After 3 h of incubation, the supernatant containing cells not adhering was transferred into a flow cytometer tube and fixed by addition of 200 μL of a 6% formalin solution. After 30 min of incubation with SYBR Green I, cell concentration was then evaluated as described above. Since the hemocyte adhesive capacity was measured with or without addition of ECP of *Vibrio aestuarianus*, results are expressed as the percentage of adhering hemocytes relatively to the initial total hemocyte concentration of the tested pool prior to incubation with or without ECP.

Reactive oxygen species (ROS) production. Measurement of the ROS production of hemocytes was assayed by modifying the procedure of Lambert *et al.* (39) using 2′7′-dichlorofluorescein diacetate (DCFH-DA). For this assay, two aliquots of 100 μL of hemolymph were diluted with 300 μL of FSSW in two flow cytometer tubes. In one of the tubes, 4 μL of diphenylene iodonium chloride (DPI, 5 μM final concentration), an NADPH-oxidase and NO-synthase inhibitor, was added. Subsequently, DCFH-DA (final concentration of 0.01 mM) was added to each tube previously maintained on ice, and tubes were incubated at 18°C for 120 min. After the incubation period, DCF fluorescence, quantitatively related to intracellular ROS production by hemocytes, was measured at 500–530 nm by flow cytometry. Results are expressed as the mean fluorescence in arbitrary units (A.U.) detected in hemocyte subpopulations maintained in FSSW (basal ROS production) and after addition of DPI (inhibited ROS production).

Statistical analysis. One- or two-way ANOVA was performed for each biochemical and hemocyte parameter using STATGRAPHICS Plus 5.1 statistical software (Manugistics, Inc., Rockville, MD), to test the ArA dose effect and the time effect after 2 and 4 wk of conditioning. Percentage data were transformed (arcsin of the square root) before ANOVA or MANOVA, but are presented in figures and tables as untransformed percentage values.

RESULTS

Condition index and biochemical composition. No significant differences between dietary treatments were observed for oyster condition index, oyster tissue dry weight (DW), or carbohydrate, protein, or lipid contents over the whole experiment (Table 1, 2-way ANOVA, $P > 0.05$). However it can be noted that oyster tissue DW and carbohydrate content were lower at the end of the experiment than initially. Meanwhile, total lipid contents of oysters increased from 0.087 mg/mg DW initially to an average of 0.110 mg/mg DW at the end of the experiment (2-way ANOVA, $P < 0.001$). Protein content of oysters was stable during the whole experiment.

FA composition of gill polar lipids: Impact of the dietary conditioning with ArA-supplemented T-Iso. Feeding oysters with T-*Iso* alone (without ArA supplementation) resulted in an enrichment of gill polar lipids with 18:1n-7, 18:1n-9, 18:2n-6, 18:3n-3, 18:4n-3, and 22:6n-3, and a decrease in

TABLE 1
Condition Index, Oyster Tissue Dry Weight, and Carbohydrate, Lipid, and Protein Contents of Oysters Fed T-*Iso* Supplemented with 0, 0.25, and 0.41 µg mL^{-1} of ArA[a]

	After 4 wk Conditioning			
	Initial	0 µg ArA mL^{-1}	0.25 µg ArA mL^{-1}	0.41 µg ArA mL^{-1}
Condition index	4.3 ± 0.9	3.6 ± 0.7	3.9 ± 0.5	3.5 ± 0.6
Oyster dry weight (mg)	0.476 ± 0.17	0.341 ± 0.07*	0.341 ± 0.15*	0.302 ± 0.09*
Carbohydrate content	0.205 ± 0.02	0.160 ± 0.02*	0.155 ± 0.01*	0.151 ± 0.02*
Lipid content	0.087 ± 0.00	0.115 ± 0.01*	0.109 ± 0.01*	0.107 ± 0.00*
Protein content	0.359 ± 0.03	0.403 ± 0.01	0.380 ± 0.02	0.393 ± 0.02

[a]All results are expressed as mean ± SD. For condition index and oyster tissue dry weight, $n = 15$. Carbohydrate, lipid, and protein contents are expressed in mg per mg oyster dry weight ($n = 3$ pools of 5 oysters). Asterisks indicate significant difference with initial composition (T-test, $P < 0.05$).

20:5n-3 (Table 2). Similar changes were observed in gill polar lipids of oysters fed ArA-supplemented T-*Iso* (Table 2), but ArA supplementation greatly increased the 20:4n-6 percentage of gill polar lipids as early as 2 wk into the dietary conditioning period (Table 2, Fig. 1). At the end of the experiment, percentages of 20:4n-6 reached 15.3% and 19.7% in gill polar lipid of oysters fed, respectively, T-*Iso* supplemented with 0.25 and 0.41 µg mL^{-1} of ArA solution, whereas it stayed at

TABLE 2
FA Composition of the Gill Polar Lipids and Total FAs of *C. gigas* Fed T-*Iso* Supplemented with 0, 0.25, and 0.41 µg mL^{-1} of ArA[a]

		After 2 wk Conditioning			After 4 wk Conditioning		
	Initial	0 µg ArA mL^{-1}	0.25 µg ArA mL^{-1}	0.41 µg ArA mL^{-1}	0 µg ArA mL^{-1}	0.25 µg ArA mL^{-1}	0.41 µg ArA mL^{-1}
FA	(n = 2)	(n = 2)	(n = 3)	(n = 3)	(n = 3)	(n = 3)	(n = 3)
14:0	0.9 ± 0.3	1.2 ± 0.0	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.3	1.0 ± 0.2
16:0	10.5 ± 0.5	9.6 ± 0.2^{a}*	9.4 ± 0.3^{b}*	8.9 ± 0.2^{b}*	9.5 ± 0.6^{a}*	8.4 ± 0.2^{b}*	8.4 ± 0.2^{b}*
18:0	5.9 ± 0.0	5.0 ± 0.2^{a}*	4.4 ± 0.2^{b}*	4.8 ± 0.1^{a}*	4.8 ± 0.4*	4.9 ± 0.3*	4.9 ± 0.2*
16:1n-7	2.2 ± 0.3	2.0 ± 0.1^{a}	1.9 ± 0.0^{b}	1.7 ± 0.0^{c}*	1.8 ± 0.1^{a}	1.3 ± 0.3^{b}*	1.1 ± 0.1^{b}*
18:1n-9	1.1 ± 0.2	2.3 ± 0.0^{a}*	1.9 ± 0.2^{b}*	1.9 ± 0.1^{b}*	2.6 ± 0.1^{a}*	2.1 ± 0.2^{b}*	2.0 ± 0.2^{b}*
18:1n-7	4.1 ± 0.2	4.9 ± 0.1*	5.3 ± 0.3*	5.4 ± 0.2*	5.2 ± 0.2*	5.3 ± 0.7*	5.5 ± 0.1*
20:1n-11	3.1 ± 0.1	2.6 ± 0.4	2.2 ± 0.3*	2.1 ±0.1*	2.0 ± 0.3*	2.2 ± 0.5*	1.9 ± 0.2*
20:1n-7	6.4 ± 0.5	5.2 ± 0.1*	5.0 ± 0.2*	5.0 ± 0.2*	5.0 ± 0.1*	5.4 ± 0.4*	5.2 ± 0.1*
18:2n-6	1.0 ± 0.3	3.5 ± 0.1^{a}*	2.7 ± 0.2^{b}*	2.7 ± 0.1^{b}*	4.0 ± 0.4^{a}*	2.8 ± 0.1^{b}*	2.6 ± 0.1^{b}*
18:3n-3	0.4 ± 0.0	0.9 ± 0.0^{a}*	0.8 ± 0.1^{ab}*	0.7 ± 0.0^{b}*	1.1 ± 0.0^{a}*	0.7 ± 0.0^{b}*	0.6 ± 0.1^{b}*
18:4n-3	0.7 ± 0.0	1.3 ± 0.0^{a}*	1.0 ±0.0^{b}*	1.0 ± 0.0^{b}*	1.4 ± 0.0^{a}*	1.1 ± 0.1^{b}*	1.0 ± 0.1^{b}*
20:2i	0.6 ± 0.0	0.6 ± 0.0^{a}	0.4 ±0.1^{b}*	0.3 ± 0.0^{b}*	0.4 ± 0.0^{a}	0.3 ± 0.0^{b}*	0.3 ± 0.0^{b}*
20:2j	0.6 ± 0.1	0.6 ± 0.0^{a}	0.6 ± 0.1^{a}	0.4 ± 0.1^{b}	0.6 ± 0.1^{a}	0.3 ± 0.1^{b}*	0.3 ± 0.1^{b}*
20:2n-6	0.3 ± 0.2	0.7 ± 0.0*	0.8 ± 0.1*	0.7 ± 0.1*	1.1 ± 0.2*	1.2 ± 0.1*	1.1 ± 0.1*
20:3n-6	0.1 ± 0.0	0.2 ±0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0*	0.3 ± 0.0*	0.3 ± 0.0*
20:4n-6	3.7 ± 0.1	3.7 ± 0.1^{a}	12.2 ± 1.4^{b}*	14.2 ± 0.3^{c}*	6.2 ± 1.5^{a}*	15.3 ± 1.0^{b}*	19.7 ± 0.5^{c}*
20:5n-3	17.1 ± 0.7	13.5 ± 0.1^{a}*	11.6 ± 0.4^{b}*	11.7 ± 0.2^{b}*	12.0 ± 1.4^{a}*	9.7 ± 0.9^{b}*	9.0 ± 0.6^{b}*
22:2i	1.7 ± 0.2	1.9 ± 0.0^{a}	1.6 ± 0.1^{b}	1.4 ± 0.0^{c}*	2.1 ± 0.4	1.7 ± 0.3	1.5 ± 0.1
22:2j	11.2 ± 0.4	8.9 ± 0.1^{a}*	7.3 ± 0.2^{b}*	7.6 ± 0.1^{c}*	7.4 ± 0.1*	7.1 ± 0.7*	6.5 ± 0.3*
22:5n-6	0.9 ± 0.2	0.8 ± 0.1^{a}	1.7 ± 0.0^{b}*	1.7 ± 0.1^{b}*	1.0 ± 0.2^{a}	2.1 ± 0.2^{b}*	2.7 ± 0.1^{c}*
22:5n-3	1.7 ± 0.2	1.4 ± 0.1	1.2 ± 0.1*	1.2 ± 0.1*	1.2 ± 0.1^{a}*	1.1 ± 0.1^{ab}*	1.0 ± 0.0^{b}*
22:6n-3	17.5 ± 0.5	21.2 ± 0.5^{a}*	18.4 ± 0.7^{b}	17.2 ± 0.4^{c}	21.3 ± 0.3^{a}*	17.2 ± 1.0^{b}	15.5 ± 0.8^{c}*
Total SFA	19.8 ± 0.8	18.2 ± 0.4^{a}*	16.9 ± 0.4^{b}*	17.2 ± 0.2^{b}*	17.4 ± 1.3*	16.1 ± 0.7*	16.4 ± 0.6*
Total MUFA	19.7 ± 0.4	19.9 ± 0.1^{a}	19.0 ± 0.2^{b}	18.8 ± 0.3^{b}	19.6 ± 0.1^{a}	19.8 ± 0.5^{a}	18.7 ± 0.4^{b}
Total PUFA	60.5 ± 1.3	61.8 ± 0.4^{a}	63.7 ± 0.8^{b}*	64.2 ± 0.5^{b}*	62.9 ± 1.1*	64.0 ± 1.1*	64.8 ± 0.9*
Total n-6	7.1 ± 0.4	9.5 ± 0.2^{a}*	18.8 ± 1.4^{b}*	21.3 ± 1.0^{b}*	13.4 ± 2.3^{a}	23.0 ± 2.0^{b}*	27.1 ± 0.2^{c}*
Total n-3	38.9 ± 1.4	39.8 ± 0.2^{a}	34.4 ± 0.7^{b}*	33.0 ± 0.4^{c}*	38.6 ± 1.0^{a}	31.4 ± 2.0^{b}*	28.7 ± 1.2^{b}*
Total NMI	14.1 ± 0.3	11.9 ± 0.1^{a}*	10.2 ± 0.1^{b}*	9.5 ± 0.3^{c}*	10.5 ± 0.1^{a}*	9.4 ± 1.0^{b}*	8.6 ± 0.3^{b}*
n-3/n-6	5.5 ± 0.5	4.2 ± 0.1^{a}*	1.8 ± 0.2^{b}*	1.6 ± 0.1^{b}*	2.9 ± 0.6^{a}*	1.4 ± 0.2^{b}	1.1 ± 0.0^{b}
20:5n-3/20:4n-6	4.6 ± 0.1	3.7 ± 0.1^{a}*	1.0 ± 0.1^{b}*	0.8 ± 0.0^{c}*	2.4 ± 0.8^{a}*	0.6 ± 0.1^{b}	0.5 ± 0.0^{c}
Total FA (µg/mg gill WW)	6.3 ± 0.3	6.3 ± 0.8	7.1 ± 0.4	7.6 ± 0.2	7.5 ± 0.2	7.2 ± 0.7	7.3 ± 0.5

[a]All results are expressed as mean ± SD. FA composition of the gill polar lipids is expressed as weight percentage of total FA in the fraction. Total FA is expressed as µg of FA per mg of gill wet weight. Different lowercase letters indicate significant differences between dietary treatments (1-way ANOVA, $P < 0.05$). Asterisks indicate significant difference with initial composition (T-test, $P < 0.05$). NMI = non–methylene interrupted FA; 20:2i = 20:2Δ5,11; 22:2i = 22:2Δ7,13; 20:2i = 20:2Δ5,13; 22:2j = 22:2Δ7,15; WW, wet weight.

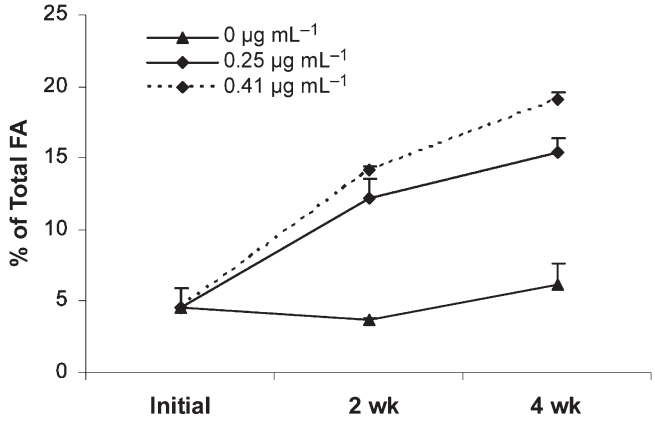

FIG. 1. ArA weight percentage of *C. gigas* gill polar lipids during the dietary conditioning (T-*Iso* supplemented with 0, 0.25, and 0.41 µg mL^{-1} ArA).

6.2% in those fed T-*Iso* alone. This high increase in 20:4n-6 also led to a significant decrease in 20:5n-3 and 22:6n-3, a significant increase in total n-6 FA contents in gill polar lipids, and consequently a decrease in the n-3/n-6 ratio and 20:5n-3/20:4n-6 ratio. However, the proportions of the different FA classes (SFA, MUFA, PUFA) were not affected by the dietary conditioning.

Impact of conditioning with ArA solution and without algae (control experiment). The FA composition of gill polar lipids was also greatly affected by 2 wk of ArA dietary conditioning with 0.25 and 0.41 µg mL^{-1} of ArA without algae supplementation (Table 3). The percentage of 20:4n-6 of gill polar lipids increased twofold after 2 wk with ArA supplementation and reached 11.3 and 12.1% in oysters fed 0.25 and 0.41 µg mL^{-1}, respectively. However, no dose effect was recorded. Similarly but to a lesser extent than in the experiment with algae, feeding oysters with 0.25 and 0.41 µg mL^{-1} also resulted in a significant decrease of 22:6n-3, a significant increase of total n-6 FA (from 9.8 to 14.5 and 15.5%, respectively) in gill polar lipids, and a significant decrease in n-3/n-6 ratio (from 4.0 to 2.2 and 2.4, respectively) and 20:5n-3/20:4n-6 ratio (from 1.7 to 0.9 and 1.0).

Hemocyte viability and concentrations. Hemocyte viability was high and similar for oysters fed T-*Iso* supplemented with 0.0, 0.25, and 0.41 ArA µg mL^{-1} (Table 4).

During the experiment, the total hemocyte (including granulocytes, hyalinocytes, and agranulocytes) tended to increase in oysters fed T-*Iso* supplemented with ArA solutions (1-way ANOVA, $P > 0.05$). When hemocyte subpopulations were evaluated, it was apparent that granulocyte and hyalinocyte concentrations of oysters fed T-*Iso* supplemented with 0.25 and 0.41 µg mL^{-1} of ArA solutions tended to be higher than those of oysters fed T-*Iso* alone (Table 4).

Percentage of phagocytic hemocytes. After 2 wk of experimental feeding, the percentage of phagocytic hemocytes from oysters fed T-*Iso* supplemented with ArA solutions was significantly higher than for oysters fed T-*Iso* alone (Figure 2, 1-way ANOVA, $P < 0.05$); however, it was not different anymore from control animals after 4 wk of dietary conditioning (1-way ANOVA, $P > 0.05$).

Adhesive capacity. After 2 and 4 wk of dietary conditioning, no change in adhesive capacity of hemocytes incubated with FSSW could be seen among the three treatment groups (average of 93% of adhering cells during the whole experiment, data not shown). However, hemocytes of oysters fed T-*Iso* alone showed a greater reduction in adhesion in response to ECP than did oysters fed T-*Iso* supplemented with ArA solutions, but this was not statistically significant (Figure 3).

Reactive oxygen species (ROS) production. ROS production by granulocytes and hyalinocytes is presented in Figure 4. A significant dietary effect was observed for the basal ROS

TABLE 3
20:4n-6 Content and Main FA Classes of the Gill Polar Lipids and Total FA of *C. gigas* Fed Different Doses of ArA Without Algae[a]

		After 2 wk Conditioning		
	Initial	0 µg ArA mL^{-1}	0.25 µg ArA mL^{-1}	0.41 µg ArA mL^{-1}
FA	(n = 3)	(n = 3)	(n = 3)	(n = 2)
20:4n-6	6.8 ± 0.3	6.7 ± 0.1[a]	11.3 ± 0.8[b]*	12.1 ± 1.2[b]*
20:5n-3	11.2 ± 0.2	11.5 ± 0.2	10.9 ± 1.0	10.4 ± 0.5
22:6n-3	24.0 ± 0.4	22.2 ± 0.6[a]*	19.9 ± 0.2[b]*	19.9 ± 0.3[b]*
Total SFA	16.7 ± 0.5	17.1 ± 0.5	17.1 ± 0.2	17.5 ± 1.1
Total MUFA	21.9 ± 0.6	21.9 ± 0.1	20.9 ± 0.2*	20.5 ± 0.1*
Total PUFA	61.3 ± 0.2	61.0 ± 0.4	62.0 ± 0.0*	62.0 ± 1.2
Total n-6	9.8 ± 0.3	9.7 ± 0.0[a]	14.5 ± 0.9[b]*	15.5 ± 1.4[b]*
Total n-3	38.9 ± 0.3	37.2 ± 0.4[a]*	34.4 ± 0.9[b]*	33.7 ± 0.3[b]*
n-3/n-6	4.0 ± 0.1	3.8 ± 0.0[a]	2.4 ± 0.2[b]*	2.2 ± 0.2[b]*
20:5n-3/20:4n-6	1.7 ± 0.1	1.7 ± 0.1[a]	1.0 ± 0.2[b]*	0.9 ± 0.1[b]*
Total FA (µg/mg gill WW)	5.5 ± 0.1	4.8 ± 0.8[a]	6.1 ± 0.5[b]	6.0 ± 0.3[b]

[a]All results are expressed as mean ± SD. FA contents are expressed as weight percentage of total FA of the fraction. Total FA is expressed as µg of FA per mg of gill wet weight. Different lowercase letters indicate significant differences between dietary treatments (1-way ANOVA, $P < 0.05$). Asterisks indicate significant difference with initial composition (T-test, $P < 0.05$). WW, wet weight.

TABLE 4
Total and Differential Hemocyte Concentrations and Percentage of Viable Cells in Oysters Fed T-*Iso* Supplemented with 0, 0.25, and 0.41 µg mL^{-1} of ArA[a]

		After 2 wk Conditioning			After 4 wk Conditioning		
	Initial	0 µg ArA mL^{-1}	0.25 µg ArA mL^{-1}	0.41 µg ArA mL^{-1}	0 µg ArA mL^{-1}	0.25 µg ArA mL^{-1}	0.41 µg ArA mL^{-1}
Total Hemocyte Counts (× 10^5 cell mL^{-1})	4.3 ± 0.6	4.3 ± 0.7	5.7 ± 1.1	6.1 ± 0.6 *	4.4 ± 1.5	5.8 ± 1.5	6.0 ± 1.1
Granulocytes	0.3 ± 0.1	0.5 ± 0.2	0.7 ± 0.2*	1.3 ± 0.0*	0.6 ± 0.2	1.0 ± 0.3*	1.1 ± 0.2*
Hyalinocytes	2.2 ± 0.3	2.6 ± 0.4	3.8 ± 0.8*	3.6 ± 0.8*	3.1 ± 1.1	3.7 ± 0.8*	3.9 ± 1.2*
Agranulocytes	1.7 ± 0.4	1.1 ± 0.2	1.2 ± 0.1	1.1 ± 0.2*	0.7 ± 0.1*	1.1 ± 0.7	1.0 ± 0.2*
Viability percentage (%)	91.6 ± 1.5	90.9 ± 2.0	90.1 ± 1.7	91.7 ± 1.3	88.7 ± 2.3	91.2 ± 1.3	89.9 ± 2.5

[a]All results are expressed as mean ± SD (n = 3). Asterisks indicate significant difference with initial composition (T-test, $P < 0.05$).

production of each hemocyte subpopulation after 4 wk of dietary conditioning (1-way ANOVA, $P < 0.05$). Granulocytes and hyalinocytes of oysters fed T-*Iso* supplemented with ArA at 0.25 and 0.41 µg mL^{-1} generated the highest basal ROS production (average of 360 A.U.). An increase of 160 A.U. for granulocytes and hyalinocytes was observed, when compared with the values obtained for oysters fed T-*Iso* alone (average of 198 A.U.). Moreover, the basal ROS production increased continuously between the initial sampling and the end of the experiment for hemocytes of oysters fed T-*Iso* supplemented with ArA solutions, whereas those of oysters fed T-*Iso* alone remained quite stable.

Regarding ROS production after DPI incubation, an inhibition of ROS production was observed for both hemocyte subpopulations, with an average of 64.5 and 56 A.U. of DCF fluorescence measured during the whole experiment in granulocytes and hyalinocytes, respectively. No dietary treatment

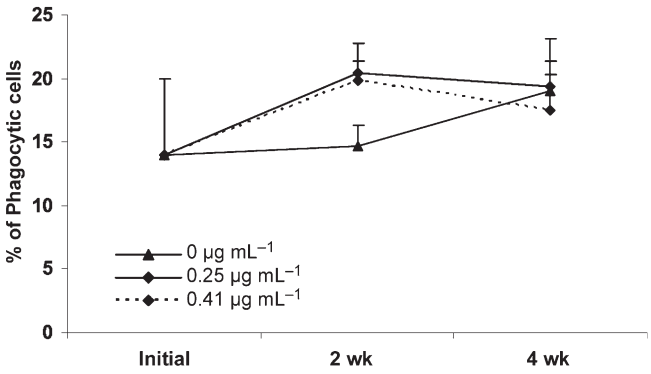

FIG. 2. Percentage of phagocytic hemocytes of *C. gigas* fed T-*Iso* supplemented with 0, 0.25, or 0.41 µg mL^{-1} ArA.

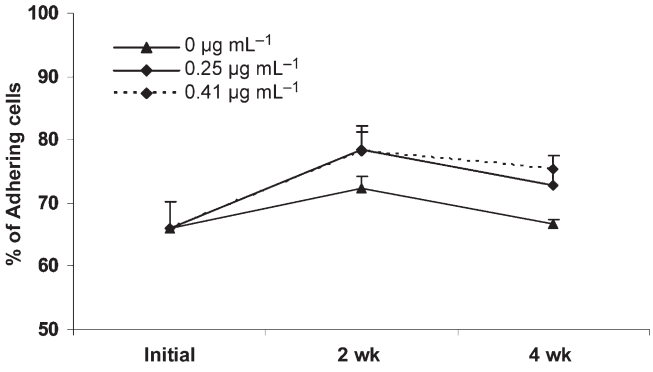

FIG. 3. Percentage of adhering cells after 3 h of incubation with 30 µg mL^{-1} of *Vibrio aestuarianus* extracellular products (ECP) prepared in seawater.

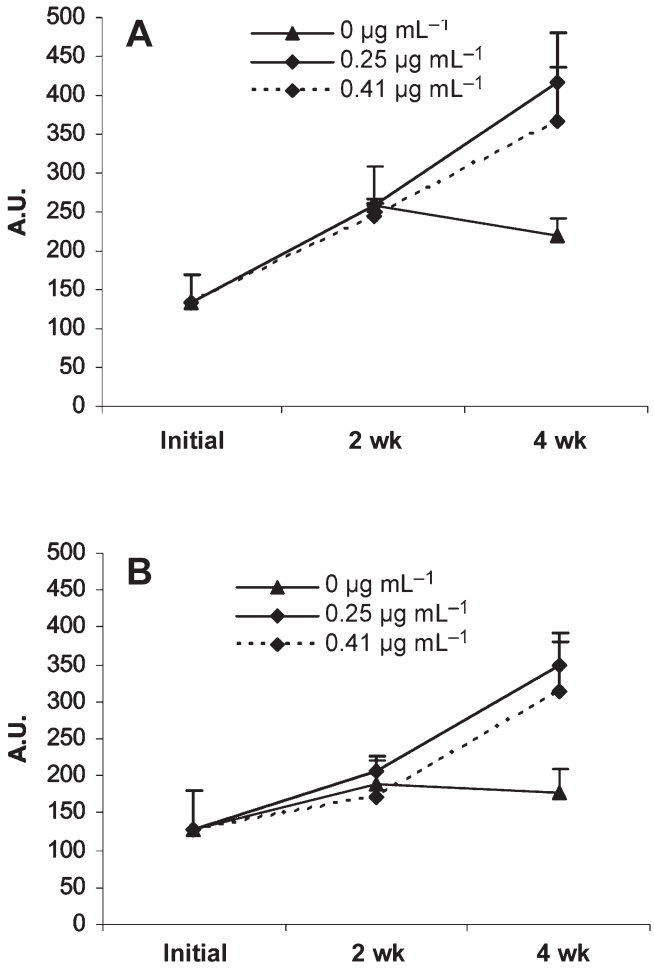

FIG. 4. Reactive oxygen species production of granulocytes (A) and hyalinocytes (B) of oysters fed T-*Iso* supplemented with 0, 0.25, or 0.41 µg mL^{-1} of ArA. Hemocytes were incubated 2 h with DCFH-DA in sterile seawater. Results are expressed in arbitrary units (A.U.).

effect was detected for each hemocyte subpopulation when incubated with DPI (2-way ANOVA, $P > 0.05$).

DISCUSSION

Impact of the dietary conditioning on gill FA composition. Changes measured in the FA composition of hemocyte membranes were shown to be closely related to those in gill membranes in dietary experiments (25); therefore, in the present study FA analyses were performed on gills rather than on hemocyte samples, which are more difficult to collect in significant amounts.

The observed changes in FA composition of gill polar lipids are in agreement with other studies using T-*Iso* as a mono-specific diet (26,40–47). More noteworthy was the increased incorporation of ArA in gill polar lipids from 4.5% to 19.7% for oysters fed 4 wk T-*Iso* supplemented with ArA solutions. Such ArA enrichment of polar lipids was also reported by Séguineau *et al.* (27) using the same technique of supplementation in *C. gigas* oyster spat for 5 wk. High concentrations of ArA in bivalve phospholipids observed in the present report and in the study of Séguineau *et al.* (27) have not been previously reported in the literature, whatever the tissue, the development stages, and species considered. The highest percentage of ArA in polar lipids found in the other literature reached 7.5% in juveniles of *C. gigas* and 10% in *Ruditapes philippinarum* (26). Also, our results confirmed that this supplementation technique greatly increased the membrane contents of polyunsaturated FA, such as ArA. Interestingly, direct ArA supplementation without algae performed in this study showed that algal feeding was not necessary to obtain good incorporation of ArA supplied in solution directly into the tanks. Indeed, increases of 40% and 44% in the ArA content in gill polar lipids were observed after 2 wk of conditioning (from 6.8% to 11.3% and 12.2%). Thus, we can suggest, as proposed by Bunde and Fried (48), that the ArA may be directly incorporated through mantle and gill membranes without passing through the digestive system. However, it must be stressed that ArA solutions were twofold more efficiently incorporated when combined with the microalgae than when provided alone. The free ArA added to the rearing tank could be coated to or be incorporated by the algae favoring thus its high incorporation and assimilation in the oyster lipids.

Concomitant with ArA incorporation, a decrease in n-3 PUFA, especially EPA content in gills, was reported during the dietary experiment, resulting in a decrease in the n-3/n-6 and EPA/ArA ratios. Although the effect of ArA supplementation on oyster immune parameters may primarily reflect the ArA content increase, the decrease of EPA content and n-3/n-6 ratio may also affect these parameters. Indeed, in vertebrates, changes in n-3 and n-6 composition and their ratio are well known to affect immune parameters. For example, EPA, by competing with ArA for the eicosanoid production, can modulate immune functions (1,2).

Impact of the ArA supplementation on immune parameters. The immune system of bivalves relies on innate defense mechanisms for its response to pathogens. The cellular component of this innate immunity is mediated by hemocytes, which are responsible for recognition, phagocytosis, and elimination of non-self particles (49–51). For this purpose, they generate a variety of microbicidal products including ROS.

In the present study, total and subpopulation hemocyte concentrations tended to be higher for oysters fed T-*Iso* supplemented with ArA. This is in agreement with increased hemocyte concentration observed in the study of Delaporte *et al.* (26) in oysters and clams fed *Chaetoceros calcitrans*, which carried the highest amounts of ArA compared with two other algae, T-*Iso* and *Tetraselmis suecica*. However, in bivalves, it is not clear whether the increase in hemocyte concentration results from production of new cells due to an ArA-enriched diet effect or migration of cells from deeper tissue into the circulating system. Moreover, comparison of our results with vertebrate immune cell proliferation must be done with caution because in vertebrates this process occurs through the binding of a mitogen to a receptor (adaptive response), which leads to a complex cascade of biochemical events. However, Peres *et al.* (52) demonstrated that ArA can modulate lymphocyte proliferation of rats according to the concentration of ArA used for loading macrophages and the percentage of macrophage in the co-culture of cells. Concomitantly, in a review, Peters-Golden *et al.* (53) underlined that leukotrienes influence innate immune response by direct effect on leukocyte accumulation, microbial phagocytosis and killing, and indirect effects mediated by elaboration of other inflammatory molecules. Consequently, although scarce references concerning eicosanoid production in bivalves exist (54–56), we propose that changes in FA composition, especially in ArA, may have affected eicosanoid metabolite production, which may be implicated in the increased hemocyte concentration observed in the present study in oysters.

With regard to hemocyte functional activities, phagocytosis by hemocytes was significantly affected by the dietary treatments after 2 wk of conditioning. At this time, the percentage of phagocytic cells of oysters fed T-*Iso* supplemented with 0.25 and 0.41 μg mL^{-1} of ArA was higher than that of oysters fed T-*Iso* alone. The highest percentage of phagocytic hemocytes was associated with the highest ArA content of gill polar lipids. The relationship between ArA and phagocytosis observed in this study may also be related to ArA-derived eicosanoid metabolites. Indeed, Canesi *et al.* (57) demonstrated that bacterial killing of hemocytes of mussel *Mytilus edulis* was reduced by inhibitors of PLA_2 and cycloxygenase activities, indicating that eicosanoid production is involved in mediating the response to bacterial challenge. Moreover, ArA metabolites (i.e., prostaglandins) were demonstrated to be involved in the nodulation process of insect hemocytes, which is the predominant response to large bacterial infections and results in the formation of an over-

lapping sheath of hemocytes around an infection target (58–63). Several *in vitro* studies in vertebrates also demonstrated a modulating effect of ArA in the phagocytosis process (3–8). Meanwhile, Brock *et al.* (64) demonstrated that LTB_4 and PGE_2 are successively expressed in macrophages during a time exposition to LPS affecting the subsequent bacterial activity of macrophage when challenged with *Klebsiella pneumoniae.* Therefore, changes in the percentage of phagocytic hemocytes observed over the ArA dietary experiment may be related to subtle changes in the ratio of ArA derived eicosanoids (i.e., LTB4 / PGE2) produced by hemocytes.

Concomitant with the increase of phagocytosis, an increase of adhesive capacity of ECP challenged hemocytes from oysters fed T-*Iso* supplemented with 0.25 and 0.41 μg mL^{-1} of ArA was observed. This result is similar to the study of Mandato *et al.* (58) and Miller *et al.* (65), who demonstrated that ArA metabolites are involved in the hemocyte spreading process (similar to the process involved in our adhesive assay) in insects and suggests that eicosanoid products may also be implicated in the adhesive capacity of oyster hemocytes. Interestingly, although the inhibitory effect of ECP on hemocyte adhesive capacity demonstrated by Labreuche *et al.* (37) was confirmed in this study, ArA supplementation appeared to counteract its effect.

An increase of the basal ROS production by hemocytes was observed at the end of the experiment for oysters fed T-*Iso* supplemented with ArA solutions. It is important to note that addition of diphenylene iodonium chloride (DPI), an inhibitor of the NADPH-oxidase and the NO-synthase pathways, strongly decreased the basal ROS production from hemocytes. This allowed us to confirm that the basal ROS production was associated with NADPH-oxidase and/or NO-synthase-like activities. Also, the highest incorporation of ArA content in gill polar lipids of those oysters seemed to be associated with basal ROS production, as previously observed with *C. calcitrans* dietary conditioning (26). In an *in vitro* study, Mazière *et al.* (66) demonstrated that human fibroblasts incubated with ArA presented higher ROS production without stimulation than control fibroblasts, but also reported a higher content of lipid peroxidation products. Also, in a review, Calder (1) noted that LTB_4 (produced from ArA) enhanced ROS production of vertebrate leukocytes. Thus, in our study, the enormous change in ArA content in gill polar lipids may have lead to an overproduction of ROS by NADPH-oxidase (with or without the production of LTB_4) and/or an increase of lipid peroxide products as in vertebrates.

CONCLUSION

In conclusion, this study demonstrated that supplementation with ArA appeared to enhance hemocyte concentration, phagocytosis activity, and basal ROS production, and to reduce susceptibility of hemocyte adhesive capacity to *Vibrio aestuarianus*. Based on established knowledge on the role of ArA and its metabolites on immune functions in vertebrates and insects, we propose that ArA and/or its metabolites may also be important in immune responses of oysters. However, additional studies are needed, and the use of inhibitors of eicosanoid synthesis should help to establish the involvement of these ArA metabolites in oyster immune parameters. Finally, as the level of ArA reported in gill polar lipids at the end of the dietary conditioning attained the highest level ever reported in literature, we wonder whether such a level of ArA is not above physiologically normal levels in oysters. New experiments with lower doses of ArA should be done, perhaps in combination with a supply of antioxidants such as vitamins E and C.

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One-Step Analysis of Major Bile Components in Human Bile Using ^{1}H NMR Spectroscopy

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ABSTRACT: Human gallbladder bile dissolved in dimethylsulfoxide provides sharp and resolved signals for major bile components in ^{1}H NMR spectra. Characteristic well-resolved marker signals that invariably appear in ^{1}H NMR spectra of bile were identified for cholesterol (H18 methyl signal at 0.643 ppm), lipids (glycerol CH signal at 5.064 ppm), total bile acids (H18 signals in the range 0.520–0.626 ppm), total glycine conjugated bile acids (NH signal at 6.958 ppm), total taurine conjugated bile acids (NH signal at 7.646 ppm), and urea (NH_2 signal near 5.48 ppm), which enabled their rapid and accurate analysis. Excellent linearity and precision of quantitative analysis was observed for all the identified bile components ($R^2 >$ 0.99 for all). The method was demonstrated on gallbladder bile from 19 patients with gallbladder diseases. Urea in bile was identified by NMR for the first time and its quantitative analysis, along with several other bile components, is presented. The majority of the bile components could be analyzed in a single step. Accurate and rapid quantification of several bile components noninvasively by using the method presented herein may have far-reaching implications in the study of bile acid metabolism and pathophysiology of various hepatobiliary and gastrointestinal diseases.

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Major components of human bile are cholesterol, glycerophospholipids, and bile acids. Altered levels of bile components are associated with various hepatobiliary and gastrointestinal diseases (1). Abnormal bile composition causes dietary fat malabsorption and gallstone formation (2,3). Hence finding bile composition is an important component for studying pathophysiology and diagnosis of diseases associated with abnormal bile composition. Several analytical methods exist for estimation of these components, but they are not straightforward and involve tedious steps such as extraction, hydrolysis, derivatization, and/or purification before analysis (4–9). Further, separate procedures need to be adapted for the analysis of individual bile components. Nuclear magnetic resonance (NMR) spectroscopy offers immense potential as a tool for investigating hepatobiliary diseases (10). The greatest advantage of this method is that several components can be monitored noninvasively, both qualitatively and quantitatively. It is the simplicity of monitoring several intact components that makes NMR a potentially useful tool in understanding disease processes and furthering the knowledge on the metabolism of bile components. Although efforts have been made to quantitatively estimate major bile components by using ^{1}H NMR spectroscopy (11,12), until today, it has not been possible to accurately analyze all of them in intact bile in a single step due to the complexity of bile spectra arising from the overlap of signals from several bile components. The analysis problem gets further compounded due to the broadening of ^{1}H NMR signals since the bile components exist as aggregates under natural conditions (13,14). In this study, we have devised a simple NMR method to analyze major bile components such as cholesterol, glycerophospholipids, total conjugated and unconjugated bile acids, and urea. This has been achieved by the study of bile in a polar organic solvent such as dimethylsulfoxide, which provides resolved NMR signals. Detailed assignments of the spectra by using 1D and 2D techniques provide means for rapid one-step analysis of several major bile components.

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Abbreviations: CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DMSO, dimethylsulfoxide-d_6; DQF-COSY, double quantum filtered correlated spectroscopy; FID, free induction decay; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TOCSY, total correlated spectroscopy; TSP, sodium salt of trimethylsilylpropionic acid-d_4.

EXPERIMENTAL PROCEDURES

Chemicals and human bile. Sodium salts of deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO). Phosphatidylcholine was obtained from Life Care Remedies and urea from Ranbaxy (New Delhi, India). Deuterated acetonitrile, deuterated acetone, deuterated dimethylsulfoxide (DMSO), deuterium oxide (D_2O), and sodium salt of trimethylsilylpropionic acid-d_4 (TSP) were purchased from Sigma-Aldrich (Milwaukee, WI). Human gallbladder bile specimens obtained from patients undergoing cholecystectomy (laparoscopic or open) for symptomatic gallstone disease were used for the experiments.

NMR experiments. All NMR experiments were performed on a Bruker Biospin Avance 400- or 700-MHz NMR spectrometer using 5-mm broadband and broadband inverse probe

heads (400 MHz) or a cryo-probe head (700 MHz) (Bruker BioSpin AG, Fällanden, Switzerland). All the probe heads were equipped with shielded *z*-gradient.

One-dimensional ^1H NMR experiments. One-dimensional ^1H spectra of bile (20 μL) dissolved in polar deuterated organic solvents (500 μL) such as DMSO, acetonitrile, and acetone were obtained by using one-pulse sequence with the suppression of the residual water signal by presaturation during the relaxation delay. In each case, bile solution was taken in a 5-mm NMR tube and the ^1H spectrum was recorded in the presence of a reusable coaxial capillary tube containing 35 μL of 0.375% TSP in D_2O.

One-dimensional ^1H spectra of individual standard chemicals, that are supposedly found in bile, such as cholesterol (51.65 μg), phosphatidylcholine (289.13 μg), urea (30 μg), a mixture of glycine-conjugated bile acids (GCA, 0.6 mg; GDCA, 0.4 mg; and GCDCA, 0.2 mg), a mixture of taurine-conjugated bile acids (TCA, 0.4 mg; TDCA, 0.2 mg; and TCDCA, 0.2 mg), and a mixture of both glycine- and taurine-conjugated bile acids (GCA, 0.6 mg; GDCA, 0.4 mg; GCDCA, 0.2 mg; TCA, 0.4 mg; TDCA, 0.2 mg; and TCDCA, 0.2 mg), were obtained in DMSO-d_6 solvent (500 μL) by using the one-pulse sequence.

Typical parameters used for all one-dimensional experiments were as follows: spectral width: 4,800 (at 400 MHz) or 9,000 Hz (at 700 MHz); time domain data points: 32 K; flip angle: 45°; relaxation delay: 6 s; spectrum size: 32 K points; and line broadening for exponential window function: 0.3 Hz.

To estimate the T_1 relaxation times of characteristic signals of individual bile component compounds, inversion recovery experiments were performed at 400 MHz on standard chemicals (cholesterol: H18; phosphatidylcholine: 2-CH; urea: NH_2; glycine conjugated bile acids: NH; taurine-conjugated bile acids: NH; and total bile acids: H18). Parameters used were as follows: spectral width: 4,800 Hz; time domain points: 32 K; relaxation delay: 15 s; number of scans: 32; inversion recovery delay: varied between 10 ms and 10 s in 10 to 13 steps; spectrum size: 32 K and line broadening: 0.3 Hz. T_1 relaxation times were calculated by using the intensities of the recovered signals with respect to the recovery delays using Bruker Xwinnmr software version 3.1.

Two-dimensional experiments. ^1H-^1H two-dimensional double quantum filtered correlated spectroscopy (DQF-COSY) and ^1H-^1H two-dimensional total correlated spectroscopy (TOCSY) experiments were performed on human bile dissolved in DMSO at both 400 and 700 MHz. Suppression of the residual water signal was done by presaturation. Parameters used were as follows: spectral width, 4,800 (at 400 MHz) or 9,000 Hz (at 700 MHz) in both dimensions; time domain data points, 2,048; number of free induction decays (FID) with t_1 increments, 512; relaxation delay, 2.5 s; and number of transients, 24. Spin lock time of 80 ms was used for TOCSY experiments. Phase-sensitive data were obtained by a time-proportional phase increment (TPPI) method. The resulting data were zero-filled to 1,024 points in the t_1 dimension and Fourier transformed along both dimensions after multiplying the data by a squared sine-bell window function shifted by π/2.

Sensitivity enhanced (by preservation of equivalent pathways using gradients) and multiplicity edited ^1H-^{13}C heteronuclear single quantum correlation (edited HSQC) and gradient enhanced ^1H-^{13}C heteronuclear multiple bond correlation (HMBC) experiments were performed at 400 and 700 MHz for bile dissolved in DMSO. Spectral widths of 4,800 (at 400 MHz) or 9,000 Hz (at 700 MHz) in ^1H dimension and 24,000 (at 400 MHz) or 44,000 Hz (at 700 MHz) in ^{13}C dimension were used; 512 FID were collected with t_1 increments each of 2,048 data points, 32 transients, and 2-s recycle delay. Phase-sensitive data for edited HSQC experiment were obtained by using echo-antiecho mode, whereas magnitude mode data were obtained for HMBC. Resulting 2D data were zero-filled to 1,024 points in the t_1 dimension and double Fourier transformed after multiplying by a squared sine-bell window function shifted by π/2 along both dimensions.

NMR experiments with titration of DMSO solution of bile with water. Bile specimens from five patients were used to test the variation of signal intensity of the bile components as a function of water content in DMSO solutions. Ten solutions were prepared from each bile specimen in DMSO in 5-mm NMR tubes varying water concentration from 1% to 10% (vol/vol) in steps of 1%, keeping the total volume of each solution fixed (500 μL). The volumes of water and DMSO in ten 500-μL solutions, from each bile, were 5, 495; 10, 490; 15, 485; 20, 480; 25, 475; 30, 470; 35, 465; 40, 460; 45, 455; or 50, 450 μL, respectively. ^1H spectra for all the solutions were recorded at 400 MHz under identical conditions by using a single-pulse sequence by suppressing water signal by presaturation. At each water-DMSO composition, amounts of cholesterol, lipids, total bile acids, glycine conjugated, and taurine conjugated bile acids were determined through the integrals of their marker signals in the spectra with reference to TSP signal (taken in a reusable coaxial capillary). Considering the maximum signal integral with reference to TSP signal as 100%, plots of integral areas of individual bile components vs. the water content were made and the variations in the NMR signal integrals were compared.

Recovery experiments for total bile acids and glycine- and taurine-conjugated bile acids. Bile from five patients (60 μL each), having different quantities of bile acids, was used for the recovery experiments. Each bile sample was divided into three parts of 20 μL each. To one part taken in a 5-mm NMR tube, 65 μL of water and 550 μL of DMSO were added, which served as a control. To the remaining two parts, a mixture of a known quantities of bile acids (DCA, 151.1 μg; CDCA, 161.3 μg; GDCA, 337.8 μg; and TDCA, 199.8 μg) were added, separately, using the bile acids stock solution, such that total volume, and composition of water and DMSO, was the same as in the control solutions. ^1H spectra were recorded for all the solutions under identical conditions by using a one-pulse sequence by suppressing the residual water signal by presaturation. Integrals of the H18 methyl signals of total conjugated and unconjugated bile acids (0.520–0.626 ppm) as well as amide signals

of total glycine (6.958 ppm) and total taurine (7.646 ppm) conjugated bile acids were determined relative to TSP reference (taken in a reusable co-axial capillary). Using these integrals, the quantities of total bile acids, glycine-conjugated bile acids, and taurine-conjugated bile acids were calculated individually from all the control spectra and those obtained after the addition of standard bile acids stock solution. The accuracy and the reproducibility of the quantities were compared by integrating each signal five times, independent of one another.

Recovery experiments for cholesterol and phosphatidylcholine. Bile from five patients (60 μL each), having different quantities of cholesterol/phosphatidylcholine as observed by the intensity of the signals, was used for the recovery experiments. Each bile sample was divided into three parts of 20 μL each. One part was taken in a 5-mm NMR tube and dissolved in 500 μL of DMSO, which served as a control. The remaining two parts were taken in separate tubes; 20 μL of stock solution containing 51.65 μg of standard cholesterol (6.68 mM) was added to each tube, and the volume was made up to 520 μL with DMSO so as to keep the total volume the same as in the control. ^{1}H spectra were recorded for all the solutions under identical conditions by using a one-pulse sequence by suppressing residual water signal by presaturation. In each case, the quantity of cholesterol was determined by using the integral of H18 methyl protons (0.643 ppm) relative to the TSP signal (taken in a reusable coaxial capillary). Similarly, following the procedure described above, recovery experiments for phosphatidylcholine were performed by adding 289.13 μg (18.92 mM) of standard phosphatidylcholine in five different bile samples (each in duplicate). In each case, the quantity of phosphatidylcholine was determined by using the integral of the glyceryl 2-CH proton (5.064 ppm) relative to the TSP reference. The accuracy and the reproducibility of the quantities were compared by integrating each signal five times, independent of one another.

Urea in bile. Urea in bile was identified through the signal at the characteristic chemical shift (near 5.43 ppm) and its variation as a function of water concentration in DMSO solution. To confirm its assignment, one-dimensional ^{1}H spectra of bile in DMSO were recorded at 400 MHz before and after the addition of standard urea (2.0, 4.0, and 6.0 μg). One-dimensional ^{13}C spectra of bile were recorded on a 700-MHz spectrometer (3,000 μL bile concentrated to 500 μL by lyophilization) with proton decoupling, before and after the addition of 0.5 mg of standard urea to further verify the assignment. The parameters used were as follows: spectral width: 44,000 Hz; time domain points: 32 K; recycle delay: 2 s; flip angle: 30°; number of transients: 7,000 (for concentrated bile) and 5,000 (for concentrated bile + 0.5 mg urea); spectrum size: 32 K; line broadening: 3 Hz;

From the titration experiments described earlier (bile solution in DMSO with water), the integral of the urea signal in the spectra of bile dissolved in DMSO containing varying amounts of water (1–10% vol/vol) was determined, to determine the solvent composition range at which the urea signal shows maximum intensity that can be used reliably for quantitative estimation. Subsequently, using the bile solution containing 8% water (vol/vol) at which the urea signal showed maximum intensity, recovery experiments were performed to determine the accuracy and precision of urea estimation. For this, six bile solutions were prepared in duplicate, each solution containing 40 μL of bile and 460 μL of DMSO. One set served as a control; to the remaining five sets, varying quantity of standard urea was added (6.96, 13.92, 20.88, 27.84, and 34.8 μg). One-dimensional ^{1}H spectra were recorded for all the samples by using a one-pulse sequence with water suppression by presaturation. The integrals of the urea signal were determined with reference to the internal reference (TSP) taken in a reusable sealed capillary. The quantities of urea recovered were calculated by using the urea signal integral after subtracting the integral from the control spectra. The accuracy and the reproducibility were tested by following the procedure as mentioned previously for other bile components.

Analyses of components in gallbladder bile. Cholesterol, lipids, total bile acids, and taurine-conjugated bile acids were determined from human gallbladder bile of 19 patients by using a simple one-dimensional ^{1}H NMR spectra obtained at 400 MHz. In each case, 20 μL of bile dissolved in 500 μL of DMSO was taken in a 5-mm NMR tube and a reusable coaxial capillary containing the quantitative reference, TSP was inserted into the NMR tube and ^{1}H spectra were obtained. A 45° radiofrequency excitation pulse and a recycle delay of 6 s were used to ensure complete recovery of magnetization of all the marker signals of bile components as well as the TSP signal. Integrals of the marker signals relative to TSP were determined and amounts of cholesterol, glycerophospholipids, taurine-conjugated bile acids, and total bile acids (conjugated and unconjugated) were calculated by using a computer program. Subsequently, 35 μL of water was added to each sample to ensure recovery of full intensity for glycine (NH) and urea signals, ^{1}H NMR spectra were recorded again, and the total glycine-conjugated bile acids and urea were determined by using the integrals of their characteristic signals with reference to TSP signal. The reproducibility of the quantities of the bile components was determined by integrating each signal five times, independent of one another.

RESULTS

Spectral assignments. The structures of major bile components that could be analyzed by NMR in this study are shown in Figure 1. Among the polar organic solvents (acetone, acetonitrile, and DMSO) used to determine appropriate conditions for obtaining resolved signals for major biochemical constituents in bile, DMSO was found to be the most suitable since it provided spectra with fairly well-resolved signals for major bile components. In acetone and acetonitrile, all the components except cholesterol were either missing or weak in intensity due to their poor solubility. ^{1}H and ^{13}C NMR signals in the DMSO solvent were identified individually based on the analysis of ^{1}H-^{1}H and ^{1}H-^{13}C two-dimensional spectra

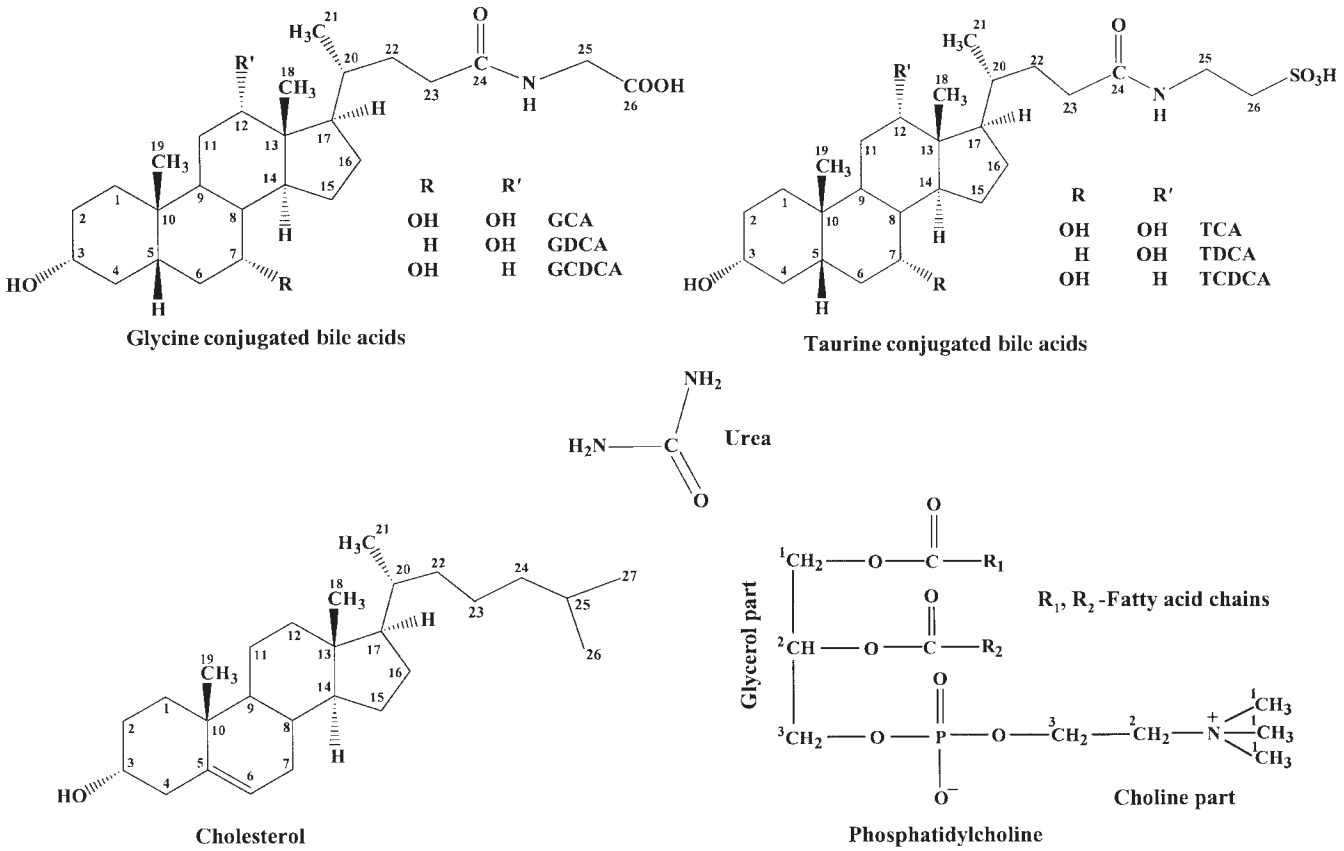

FIG. 1. Structures and numbering of protons and carbons of bile components dealt with in this study. The numbering of the proton(s) is the same as that of the corresponding directly attached carbon. The orientation of some substitutions in cholesterol and bile acids is marked with respect to the plane of the ring (crosshatched wedge, α substitution; bold wedge, β substitution).

obtained at 400 and 700 MHz and using the library of chemical shifts recently established for several conjugated and unconjugated bile acids (15). Parts of TOCSY, HSQC, and HMBC spectra along with the assignments of major signals are shown in Figures 2, 3, and 4, respectively. Although, ^{1}H NMR signals were relatively sharp in DMSO, many of the signals were still overlapping, obviously due to the presence of several components (Fig. 5). However, the methyl signal of cholesterol (H18, 0.643 ppm), the methyl signals of total bile acids (H18, 0.520–0.626 ppm), the glyceryl 2-CH signal of phosphatidylcholine (5.064 ppm), and the amide signals of glycine-conjugated (6.958 ppm) and taurine-conjugated (7.646 ppm) bile acids were invariably distinctly isolated from each other and from the rest of the signals in the ^{1}H NMR spectra. One-dimensional ^{1}H spectrum of a typical bile sample dissolved in DMSO (with assignments) is shown in Figure 6 along with the spectrum of neat bile diluted with water for comparison.

Bile signals intensity vs. water content in DMSO solution. In the spectrum of bile dissolved in DMSO, the intensities of signals from cholesterol, lipids, and amide signals of bile acids vary with the composition of water. Both cholesterol and lipids show maximum signal intensity in DMSO up to a water composition of about 4% (vol/vol). However, beyond 4% (vol/vol), the signals gradually broaden and eventually become undetectable (Fig. 7). Methyl signals of total bile acids and amide signals of taurine-conjugated bile acids show maximum intensity throughout the range of water-DMSO composition studied. Amide signals from glycine-conjugated bile acids show maximum intensity in DMSO containing ≥ 6% water (vol/vol). Consistent results obtained for bile specimens from five different patients are depicted for all the bile components in Figure 7.

Urea in bile. Urea in bile was initially tentatively assigned from the characteristic ^{1}H signal at 5.48 ppm (in DMSO solution containing 10% water). The urea signal overlaps with the unsaturated part of lipids signal (~5.31 ppm) in the ^{1}H NMR spectrum of bile in DMSO containing around 1% (vol/vol) water. However, as the water content in DMSO is increased, the signal gradually shifts to downfield. When the water content was increased to more than 3% (vol/vol), the signal separates out of the overlapping region. Parts of a few spectra showing the variation of urea chemical shift with reference to water content are shown in Figure 8. One-dimensional ^{1}H and ^{13}C NMR spectra recorded before and after the addition of standard urea into the bile solution clearly showed, after addition of urea, increase in signal intensities of both ^{1}H and ^{13}C signals of urea (Fig. 9), thus confirming the assignment of bile urea made for the first time in bile NMR spectrum.

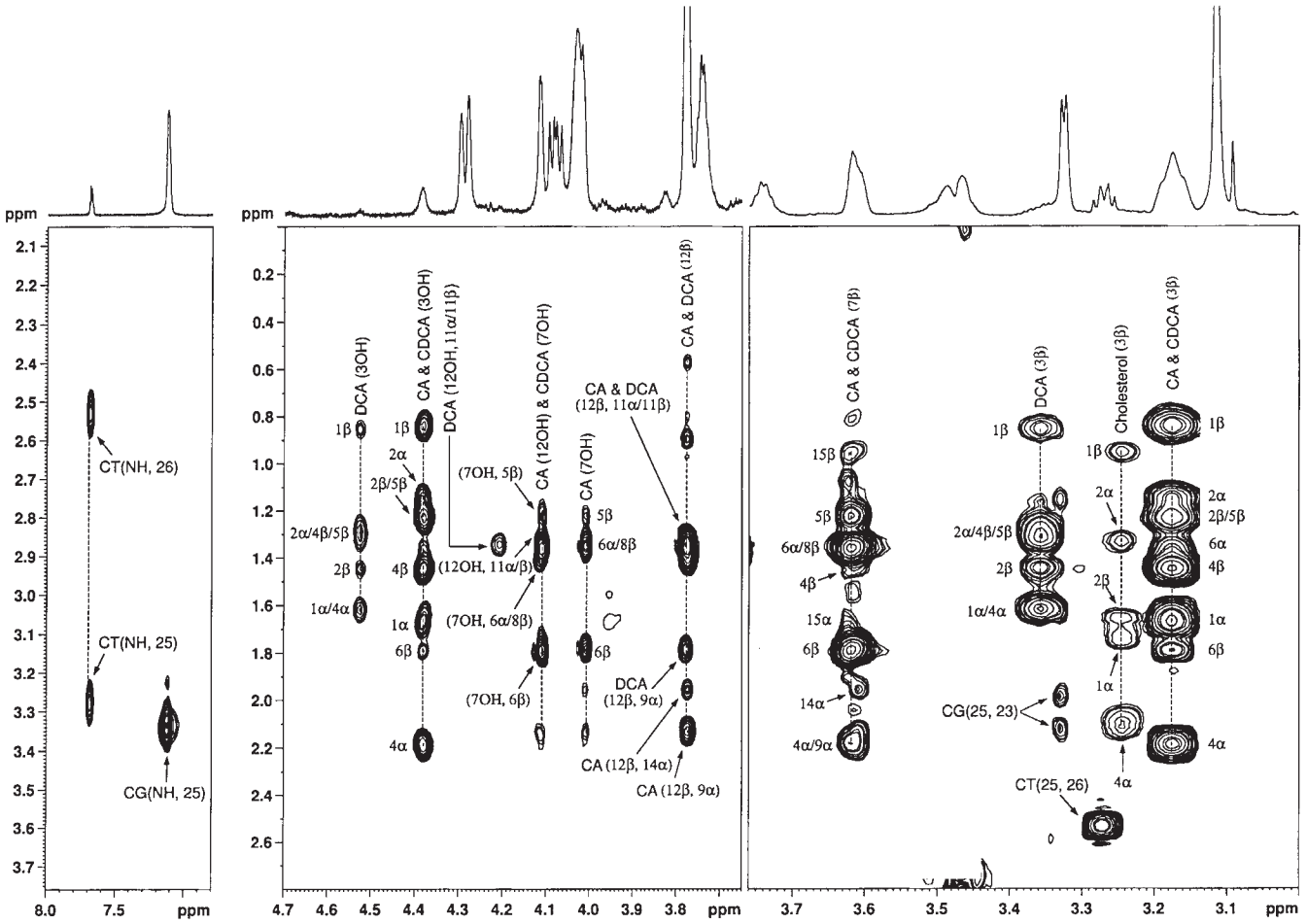

FIG. 2. Part of TOCSY spectrum of typical bile (20 µL) dissolved in 500 µL of DMSO with assignments of the signals. The spectrum was obtained on a Bruker Biospin AVANCE 700-MHz spectrometer (Bruker BioSpin AG, Fällanden, Switzerland). CT, conjugated taurine; CG, conjugated glycine; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxy cholic acid. The numbering of protons is as shown in Figure 1. A trace of the 1D ^{1}H spectrum is shown on the 2D spectrum. The residual signals (T_1 noise) arising from the water peak at 3.5 ppm and phosphatidylcholine peak at 3.1 ppm are whited out for the sake of convenient labeling of the cross peaks (rightmost part of the spectrum).

A one-dimensional experiment on bile dissolved in DMSO with water varying from 1% to 10% (vol/vol) showed that, at a lower concentration of water, the urea signal intensity is lesser and it gradually increases as the concentration of water is increased, and the intensity was maximum (100%) at greater than a 7% (vol/vol) water concentration. Plots of urea signal integral vs. water composition for five different bile specimens dissolved in DMSO are included in Figure 7.

Accuracy and precision tests. The accuracy and precision of analysis of cholesterol, lipids, total bile acids, conjugated bile acids, and urea were determined from NMR of bile in DMSO by using recovery experiments. Comparison of the quantities of biochemical constituents recovered, in duplicate experiments, from intact bile in DMSO with the quantities added showed excellent correlation ($R^2 > 0.99$) for all the bile components.

Rapid analysis of human gallbladder bile. It is clear from Figure 7 that ^{1}H NMR spectra of bile in DMSO containing 4% or less (vol/vol) water show isolated signals with maximum intensity for cholesterol, lipids, total bile acids, and taurine-conjugated bile acids. This establishes that the signal intensities in DMSO containing 4% or less (vol/vol) water for cholesterol, lipids, total bile acids, and taurine-conjugated bile acids represent their true concentrations. Under these conditions (20 µL of bile dissolved in 500 µL of DMSO), cholesterol, lipids, total bile acids, and taurine-conjugated bile acids were estimated simultaneously in bile from 19 patients with gallbladder disease. Subsequently, glycine-conjugated bile acids and urea were estimated from the spectra obtained after the addition of 35 µL of water. In all the bile specimens, bile components appear consistently in the ^{1}H NMR spectra; however, the quantities as well as the relative composition of the components varied significantly. Table 1 shows the absolute quantities of various components thus determined from ^{1}H NMR for gallbladder bile of 19 patients. Parts of ^{1}H NMR spectra for bile from four representative patients are shown in Figure 10, highlighting variation of total and relative amounts of various components. The diagnosis

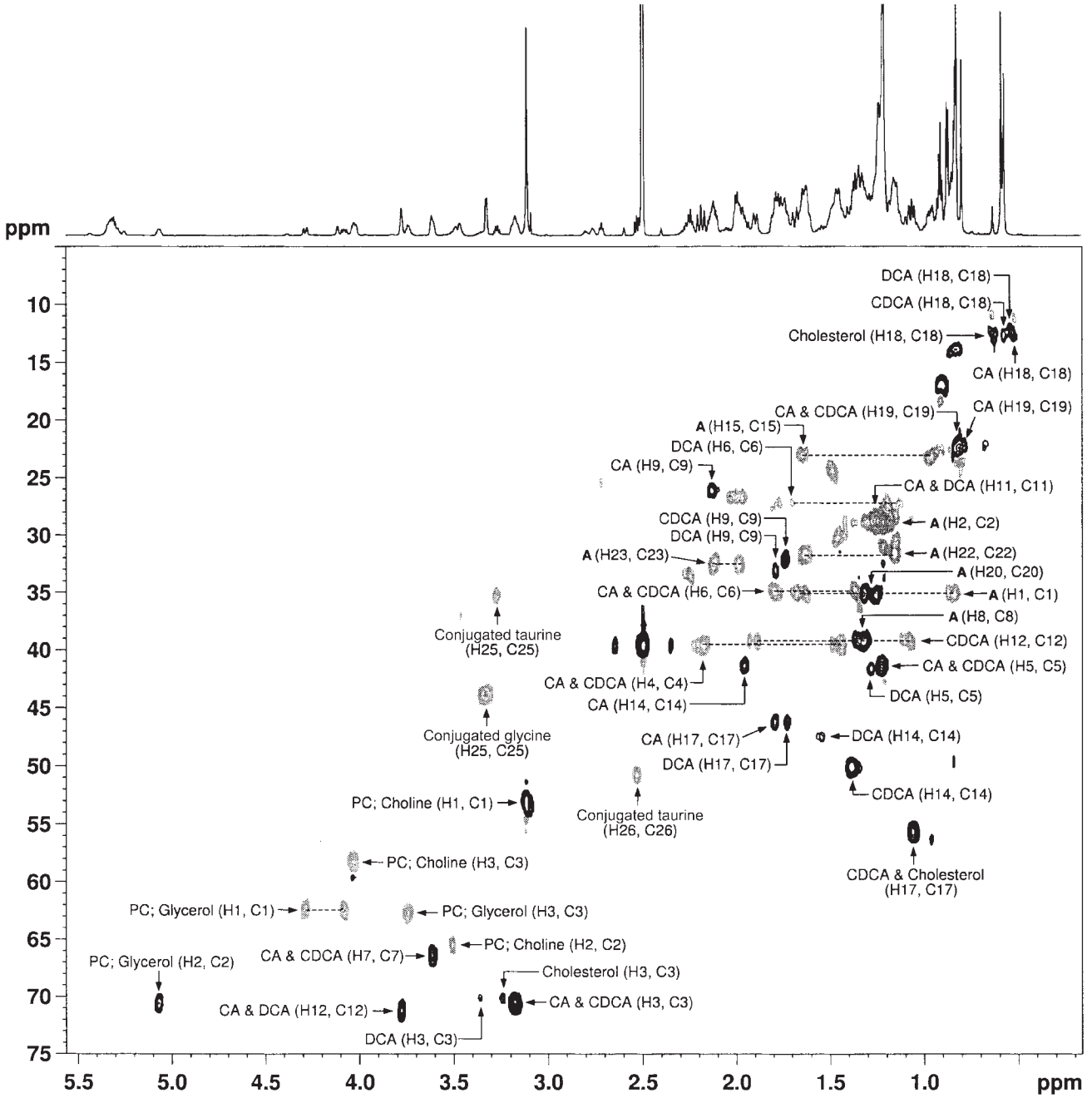

FIG. 3. Sensitivity-enhanced multiplicity edited ^{1}H-^{13}C HSQC spectrum of 20 µL of bile dissolved in 500 µL of DMSO obtained on a Bruker Biospin Avance 700-MHz spectrometer, along with the assignments of the cross peaks of protons and the attached carbons. For clarity, CH and CH_3 cross peaks are shown as dark continuous contours, whereas CH_2 cross peaks are shown in light discontinuous contours. Wherever the two protons of CH_2 are chemically different (nondegenerate), two distinct cross peaks were observed corresponding to H_α and H_β protons (each pair of cross peaks is joined by dashed lines). A trace of the 1D ^{1}H spectrum is shown on the 2D spectrum. PC, phosphatidylcholine; CA, glycine/taurine conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxycholic acid. A: All glycine/taurine-conjugated bile acids. The numbering of the protons/carbons is as shown for the structures in Figure 1.

of the gallstone disease for each patient is also included in the Table.

DISCUSSION

Biochemical composition of bile reflects a multitude of cellular functions and hence it is important that a convenient method exists to monitor the dynamic variation of several of the bile components simultaneously. NMR spectroscopy happens to be a potentially useful tool for such a purpose. In an effort to quantify total glycine- and taurine-conjugated bile acids in bile, we have recently proposed a simple pulse NMR method (16). In this study, using polar organic solvents such as deuterated acetone, acetonitrile, and DMSO, we have attempted to simplify

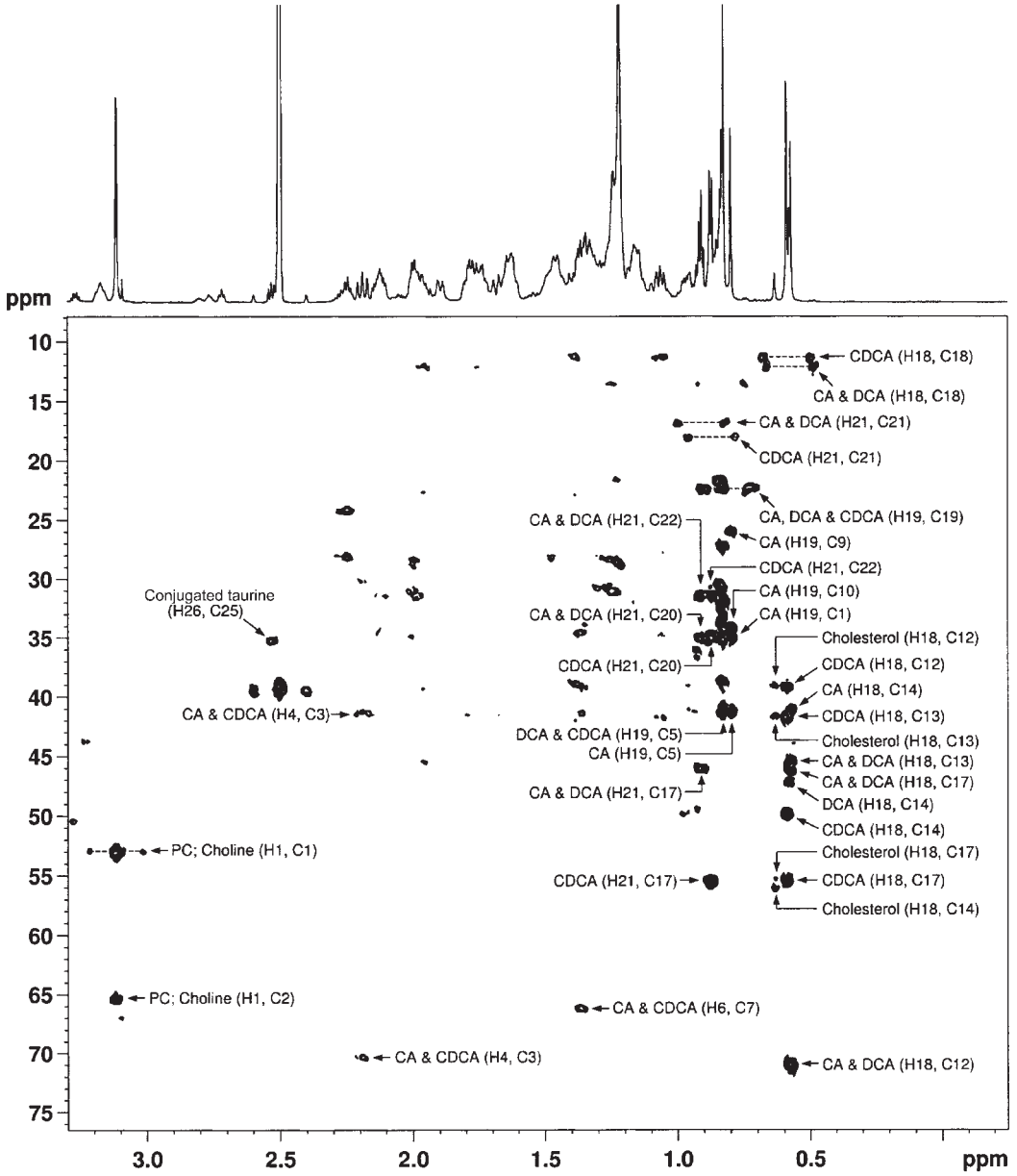

FIG. 4. ^{1}H-^{13}C HMBC spectrum of 20 µL of bile dissolved in 500 µL of DMSO obtained on a 700-MHz Bruker Biospin AVANCE spectrometer, along with the assignments of the cross peaks arising from one-, two-, or three-bond ^{1}H and ^{13}C couplings. Cross peaks due to one-bond proton-carbon coupling are joined by horizontal dashed lines. A trace of the 1D ^{1}H spectrum is shown on the 2D spectrum. PC, phosphatidylcholine; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxy cholic acid. The numbering of the protons/carbons is as shown for the structures in Figure 1.

the complex human bile spectra to analyze several bile components, simultaneously, without resorting to any complex procedure. The signals from all bile components were not clearly observed in the spectra of bile in acetone or acetonitrile due to the poor solubility of especially urea and bile acids. However, the spectra of bile dissolved in a highly polar solvent, such as DMSO, provided signals with the characteristic marker signals for major bile components such as cholesterol, glycerophospholipids, total bile acids (conjugated and unconjugated), total glycine-conjugated bile acids, and total taurine-conjugated bile acids well separated from each other, which enabled their accurate quantitative analysis (Figs. 6 and 10). It may be noted that 95% of the phospholipids in the bile arise from phosphatidylcholine (17), and hence the contributions from other lipids are minor. Further, the 2-CH signal (at 5.064 ppm) couples with 1-CH_2 and 3-CH_2 of glycerol moiety, all have chemical shifts typical for phosphatidylcholine as observed in 2D DQF-COSY, TOCSY, and HSQC spectra (Figs. 1 and 3), and hence the 2-CH signal used as a marker for glycerophospholipids, in this study, arises purely from phosphatidylcholine. Identification of the bile components marker signals was, first, based on the assignment of ^{1}H and ^{13}C signals using one- and

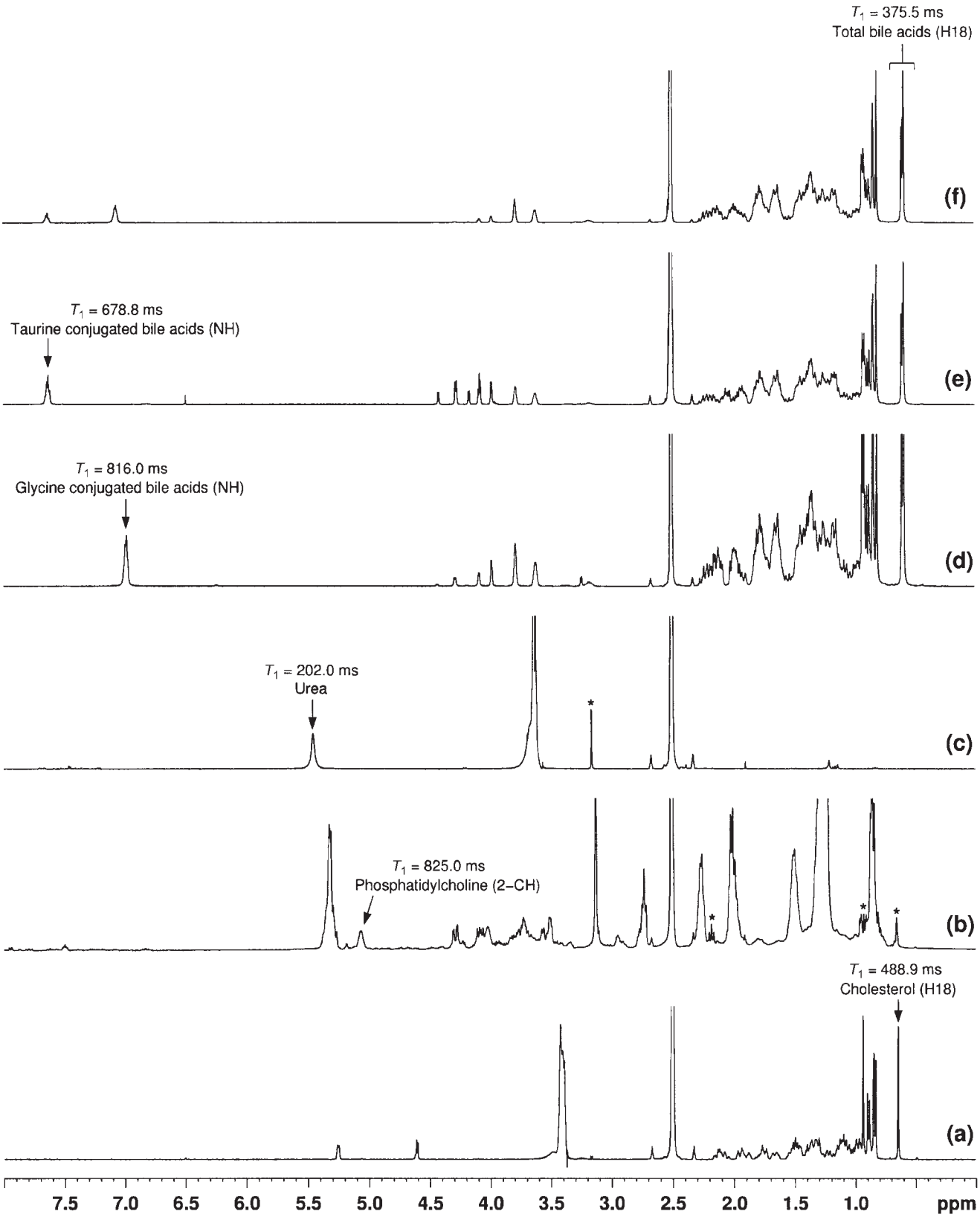

FIG. 5. One-dimensional ^{1}H spectra of individual bile component compounds obtained on a 400-MHz Bruker Biospin AVANCE spectrometer in DMSO solvent (500 µL): (a) cholesterol (51.65 µg); (b) phosphatidylcholine (289.13 µg); (c) urea (30 µg); (d) mixture of glycine-conjugated bile acids (GCA: 0.6 mg; GDCA: 0.4 mg; and GCDCA: 0.2 mg); (e) mixture of taurine-conjugated bile acids (TCA: 0.4 mg; TDCA: 0.2 mg; and TCDCA: 0.2 mg); (f) mixture of taurine- and glycine-conjugated bile acids (GCA: 0.6 mg; GDCA: 0.4 mg; GCDCA: 0.2 mg; TCA: 0.4 mg; TDCA: 0.2 mg; and TCDCA: 0.2 mg). The characteristic marker signal(s) and their T_1 values are marked. * = impurity signals.

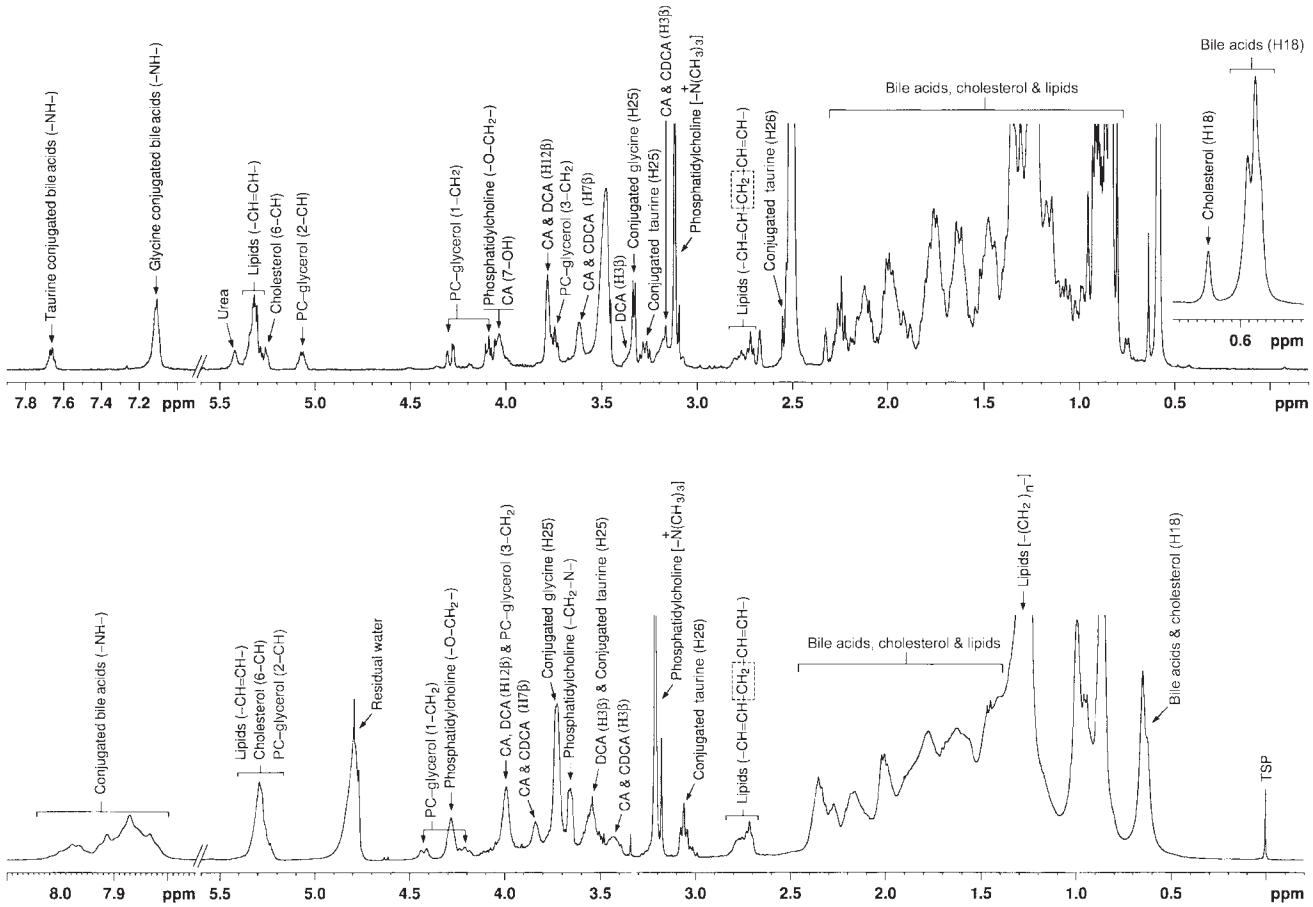

FIG. 6. One-dimensional ^{1}H spectra obtained on a 400-MHz Bruker Biospin AVANCE spectrometer. (Top) Spectrum of 20 µL of bile dissolved in 500 µL of DMSO with the assignments of the majority of the signals. Characteristic marker signals for cholesterol, lipids, total bile acids, glycine-conjugated bile acids, taurine-conjugated bile acids, and urea are distinctly observed. (Bottom) Bile spectrum in aqueous medium (100 µL of bile in 400 µL of water); note that the marker signals for individual classes of bile components are overlapping, thus making it difficult (or impossible) for analysis of bile components in the spectra from aqueous medium. PC, phosphatidylcholine; CA, glycine/taurine conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxy cholic acid. The numbering of protons is as shown for the structures in Figure 1.

two-dimensional experiments (Figs. 2–4). Initially, partial assignments were made by using the NMR experiments at 400 MHz and the library of chemical shifts of several bile acids (15). Subsequent assignments using the spectra at 700 MHz greatly facilitated unambiguous assignment of the majority of ^{1}H and ^{13}C signals.

Quantitative estimation from ^{1}H NMR spectroscopy requires that at least one of the proton signals is separated from the crowded regions of the spectra. Even from the regions of the spectra with signals overlapping from several components, it is still possible to quantify them accurately provided the number of protons of each constituent in the overlapping signals is known and it is same for all the components. In the spectra of bile dissolved in DMSO, H18 methyl signals of total bile acids (conjugated and unconjugated) at 0.520–0.626 ppm, the H18 methyl signal of cholesterol at 0.643 ppm, glyceryl 2-CH of lipids at 5.064 ppm, urea amide signals near 5.48 ppm (in the presence of nearly 8% water), amide signals of total glycine-conjugated bile acids at 6.958 ppm, and amide signals of total taurine-conjugated bile acids at 7.646 ppm are the distinct markers of the individual class of components, which enabled their accurate analyses (Figs. 6 and 10). To choose an appropriate recycle delay for accurate quantitative measurement of all the components in a single measurement, we have estimated T_1 relaxation times for the key resonances by using an inversion recovery method and the values thus obtained are marked in Figure 5. It may be noted that the relaxation times of all the key resonance lie well within 1 s and hence favor quick acquisition of the NMR data. However, care should be taken while analyzing bile components in a single step since water content in DMSO greatly alters the intensities of several bile components signals. This is particularly important since bile itself is an aqueous medium, and, depending on the volume of bile dissolved in DMSO, the amount of water will automatically vary, which directly influences the outcome of the results. After a careful study of bile dissolved in DMSO with different amounts of water arising from the dissolution of different volumes of bile, we have

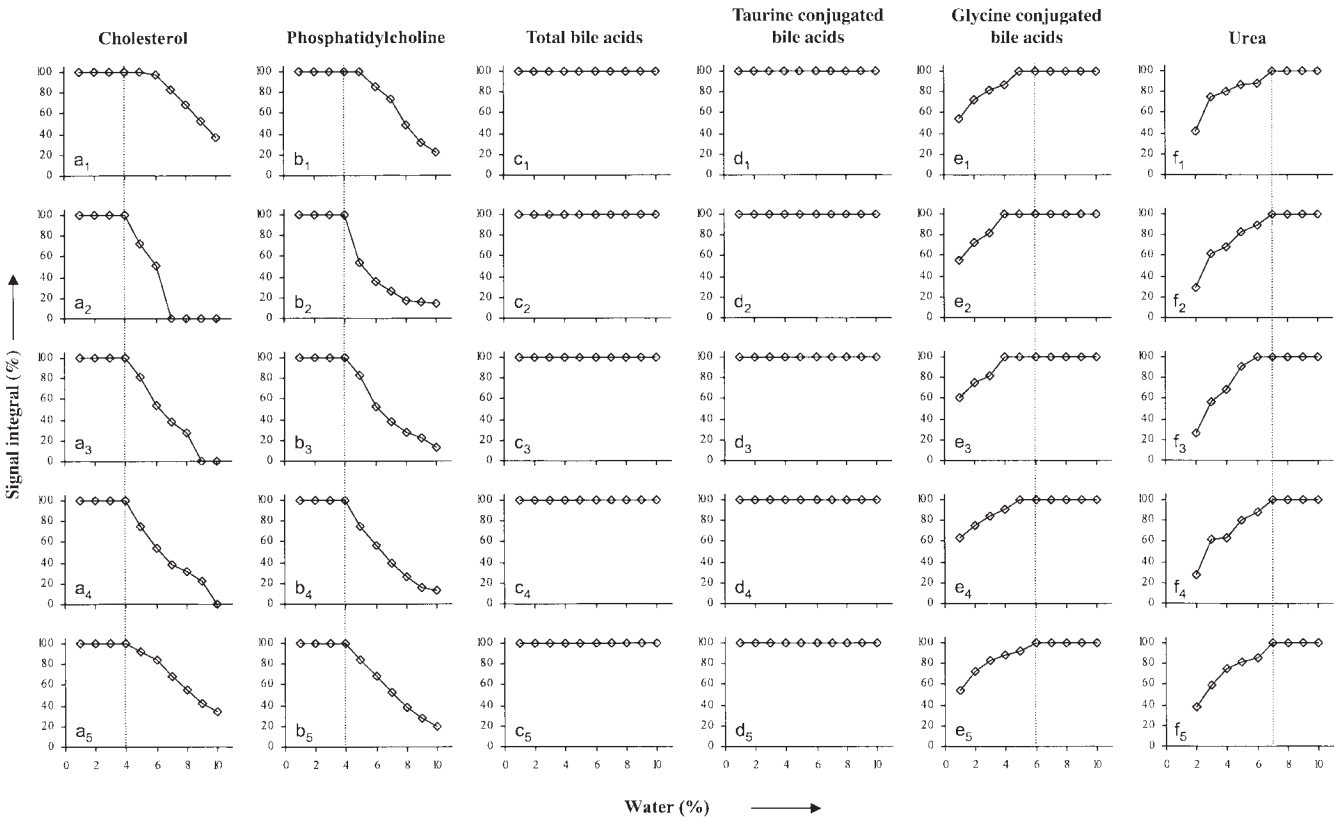

FIG. 7. Plots of intensity of signals vs. percentage of water in DMSO solution of bile studied for five patients' bile; 100% refers to the true integral corresponding to the actual quantity of the biochemical present in the solution. Clearly, maximum signal intensity is obtained for cholesterol (a_1 to a_5) and lipids (b_1 to b_5) when water is 4% (vol/vol) or less; for total bile acids (c_1 to c_5) and taurine conjugated bile acids (d_1 to d_5), the signal is maximum throughout; for glycine conjugated bile acids (e_1 to e_5) and urea (f_1 to f_5), the signal integral becomes maximum beyond nearly 6% and 7% (vol/vol), respectively.

shown that to analyze cholesterol and lipids along with total bile acids and taurine-conjugated bile acids, in a single step, the water content in DMSO must be limited to 4% (vol/vol) or less (Fig. 7). This condition is stringent for cholesterol and lipids analyses since their signal intensities drastically decrease at higher than 4% (vol/vol) water. This reduction in intensity presumably arises from the aggregation of the predominantly nonpolar lipid/cholesterol molecules. Thus, dissolution of about 20 µL or less of bile in about 500 µL of DMSO would satisfy the condition (water: 4% [vol/vol] or less) for rapid analysis of the components. As shown from this study, about 20 µL of bile is sufficient to obtain spectra with a good signal-to-noise ratio within a few minutes even with lower field NMR spectrometry (400 MHz).

Attenuation of the amide signal intensity of glycine-conjugated bile acids in DMSO containing less than 6% (vol/vol) water (Fig. 7) may arise from the interaction of the amide protons with the DMSO solvent. Upon increasing water concentration to greater than 6% (vol/vol), an attenuated glycine amide signal gains its full intensity, which appears to be due to the competing interaction of water with DMSO. Hence, for accurate analysis of total glycine-conjugated bile acids, an additional ^{1}H NMR experiment is required after increasing the amount of water to 6% (vol/vol) or more. However, it may be noted that bile acids constitute mainly glycine- and taurine-conjugated bile acids (4). In addition, our study on a large number of bile specimens from different patients showed the ratio of the sum of glycine and taurine amide signal integrals to the total bile acids H18 methyl signal integral to be 1:3, as expected for bile acids constituted by only glycine and taurine conjugates. It can also be seen from Table 1 that the total bile acids determined by using H18 methyl signals are nearly equal to the respective sum of glycine- and taurine-conjugated bile acids determined from the amide signal integrals. Under such conditions, an additional NMR experiment is not required for analyzing glycine-conjugated bile acids and it can be determined along with other bile components by subtracting total taurine-conjugated bile acids from the total bile acids (Table 1). On the other hand, after determining glycine-conjugated bile acids accurately from an additional experiment, unconjugated bile acids present in the bile, if any, may also be determined accurately by simply subtracting the sum of the glycine- and taurine-conjugated bile acids from the total bile acids. However, care should be used while interpreting such results since, similar to unconjugated bile acids, sulfonated or glucuronated (18,19) bile acids, if present in significant quantity, would also result in the increase of total bile acids marker signals.

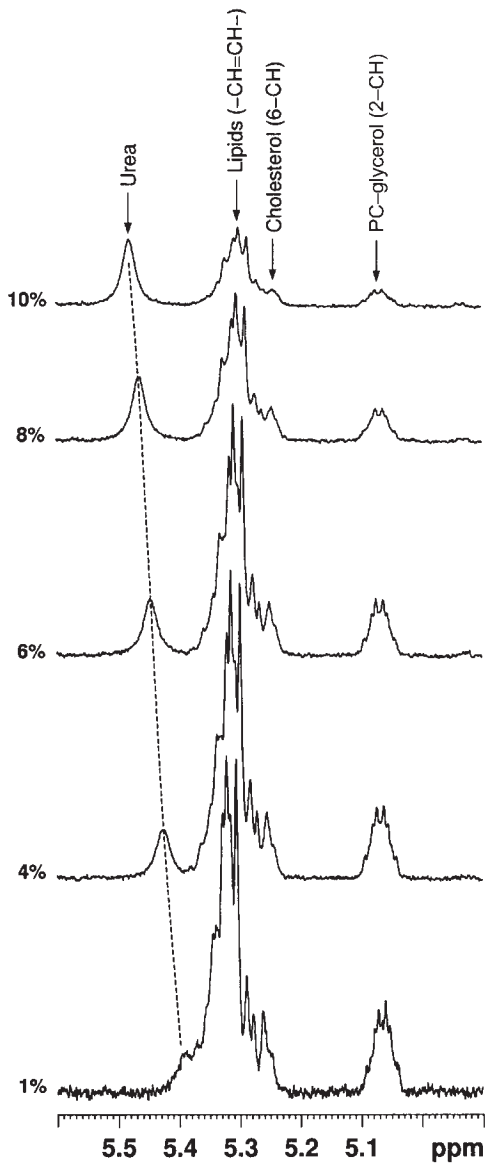

FIG. 8. Parts of ^{1}H NMR spectra of bile dissolved in DMSO, obtained on a 400-MHz spectrometer, highlighting the urea signal in bile and its variation (shifting toward higher chemical shift) of position as the water in the DMSO solution is increased from 1% to 10% (vol/vol). Note that the intensity of the urea signal also increases at higher percentages of water.

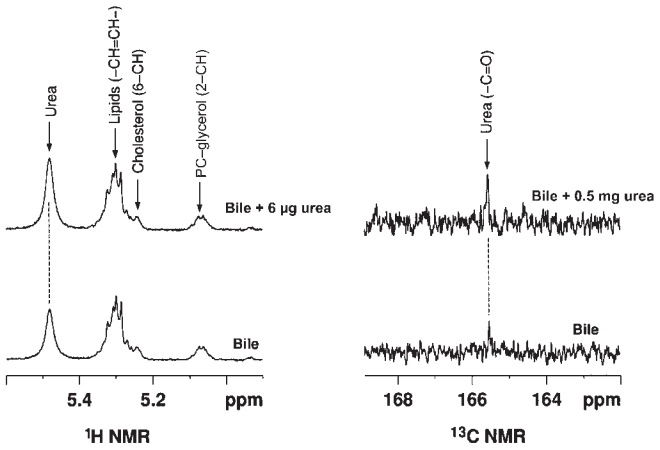

FIG. 9. (Left) Parts of 400-MHz ^{1}H NMR spectra of 20 µL of human bile dissolved in 500 µL of DMSO (containing 8% water) obtained under identical conditions before (bottom spectrum) and after (top spectrum) the addition of 6 µg of urea. (Right) Parts of 700-MHz ^{13}C NMR spectra of 3,000 µL of human bile concentrated to 500 µL obtained before (bottom spectrum) and after (top spectrum) the addition of 0.5 mg of urea. Note that the urea signal in both ^{1}H and ^{13}C NMR spectra increases after the addition of urea, thus confirming its identification, for the first time, in bile by NMR. More noise in the ^{13}C spectrum (top right spectrum) arises due to a fewer number of transients (5,000 transients) when compared with the number of transients for the control spectrum (7,000 transients; bottom right spectrum). The vertical scales for all the spectra are normalized with reference to the internal invariant bile acids signals.

The accuracy and precision of analysis of cholesterol, lipids, total bile acids (conjugated and unconjugated), and glycine-conjugated and taurine-conjugated bile acids were determined from the intact bile dissolved in DMSO before and after the addition of known quantities of standard compounds and showed good correlation ($R^2 > 0.99$). This accuracy of the analysis for bile components using ^{1}H NMR essentially stems from the fact that the characteristic marker signals for bile components in DMSO are well resolved, which enables measurement of the integrals of the characteristic signals precisely.

In this study, we also report the identification and quantitative estimation of urea in bile for the first time by using NMR. In DMSO with less than about 2% (vol/vol) water, the urea signal overlaps with the unsaturated part of lipids signals. However, with increasing water content, the urea signal gradually separates out from the overlapping region with a concomitant increase of the intensity. Reduced intensity at a lower concentration of water, similar to the amide signal of glycine-conjugated bile acids, may arise from the interaction with the DMSO solvent. This interaction of urea with DMSO appears to gradually diminish with increasing water content, apparently due to the competitive interaction of water with DMSO. The quantity of urea in bile analyzed before and after the addition of varying quantities of standard urea at a water-DMSO composition at which the signal intensity was maximal provides excellent precision and linearity.

The water signal in the DMSO solution of bile appears at about 3.5 ppm. Care should be used during the suppression of this signal because high water suppression power would attenuate nearby signals. The suppression power might even spread to the characteristic lipid and urea marker signals (near 5 ppm, Figs. 6 and 10), depending on the strength of the radiofrequency power used, and cause underestimation of lipids and urea. However, the suppression power of about 10 Hz or less is safe and sufficient to suppress the residual water signal without causing attenuation of required component signals.

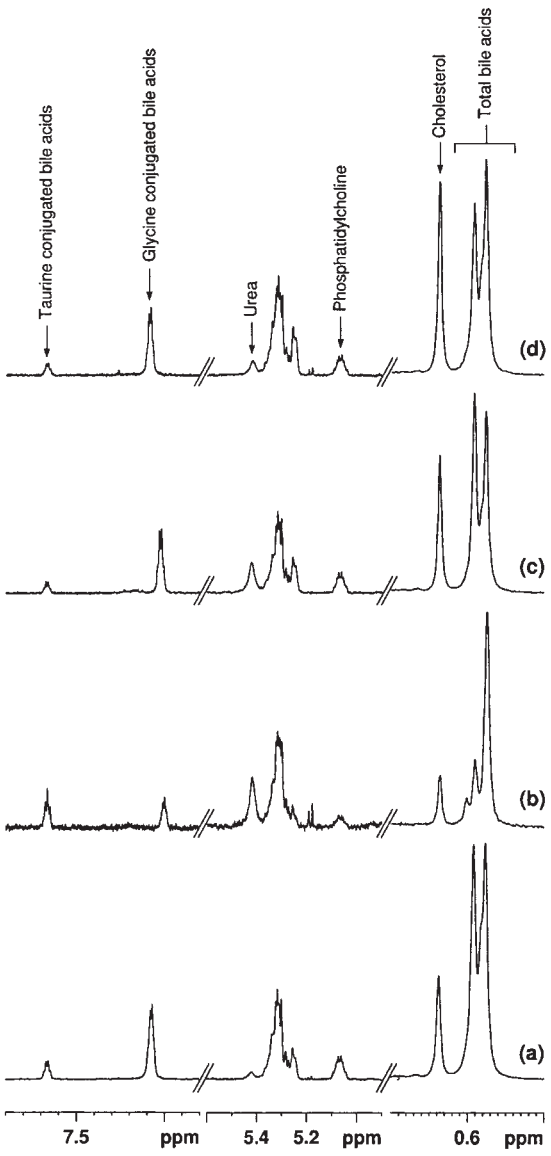

FIG. 10. Parts of 400-MHz ^{1}H NMR spectra of gallbladder bile (20 µL) dissolved in 500 µL of DMSO from four typical patients with gallbladder disease highlighting the isolated characteristic marker signals of several major bile components that can be accurately and rapidly analyzed. Variation of the relative intensities of the bile components within as well as between the bile specimens is clearly noticeable (see also Table 1). All the spectra were acquired and plotted under identical conditions for direct comparison of the relative quantities of various bile components. The regions 4.9 to 5.6 ppm and 6.8 to 7.9 ppm are plotted with increased vertical scale by a factor of 2 with respect to the region 0.5 to 0.7 ppm. (a) Normal (no diagnosed gallstone disease); (b) xanthogranulomatous cholecystitis (XGC); (c) gallbladder cancer (GBC); and (d) chronic cholecystitis (CC).

We have demonstrated simultaneous analysis of several bile components from gallbladder bile from 19 patients. As seen in Figure 10 and Table 1, significant differences in the absolute as well as the relative quantities of the bile components for these patients indicate the potential application of the method for better understanding of bile biochemistry. Further, in all the bile specimens, the amount of total bile acids determined from H18 methyl signals was nearly equal to the sum of total glycine- and taurine-conjugated bile acids (Table 1). This is in line with the fact that glycine and taurine conjugates are the predominant bile acids in bile (18). Although variation in the quantities of the individual bile components, as shown in Table 1, does not seem to show any direct correlation with the diagnosis, detailed and systematic studies are required to relate these variations to the underlying pathophysiology of hepatobiliary diseases.

In summary, we present here a simple method to analyze several major bile components such as cholesterol, lipids, total bile acids (conjugated and unconjugated), total glycine-conjugated bile acids, and total taurine-conjugated bile acids. This method allows analyses of major bile components such as cholesterol, lipids, and total bile acids, including taurine-conjugated bile acids, in a single step. An additional measurement with a few microliters of water will enable the analyses of glycine-conjugated bile acids and urea. Urea in bile was identified for the first time by using NMR. The whole process is so rapid that within a few minutes, all the components can be analyzed. This rapid and noninvasive method enables quick monitoring of dynamic variations of the bile components, providing greatly simplified access for better understanding of hepatic cell functions, bile synthesis, and the associated biliary diseases.

ACKNOWLEDGMENTS

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TABLE 1
Bile Components Determined from Intact Gallbladder Bile Diluted with Water of 19 Patients Using the Described Rapid NMR Method[a]

Bile sample	Cholesterol	Lipids	Total bile acids	Glycine conjugated bile acids	Taurine conjugated bile acids	Urea	Disease type
1	24.77 ± 0.13	39.18 ± 0.19	145.11 ± 0.49	119.11 ± 0.93	24.63 ± 0.16	2.60 ± 0.06	XGC
2	3.22 ± 0.02	6.86 ± 0.11	26.91 ± 0.27	22.48 ± 0.23	5.26 ± 0.08	2.19 ± 0.04	XGC
3	1.17 ± 0.04	3.88 ± 0.11	17.11 ± 0.37	13.21 ± 0.16	4.39 ± 0.07	2.35 ± 0.06	XGC
4	1.10 ± 0.04	ND	2.06 ± 0.06	ND	ND	2.13 ± 0.05	XGC
5	23.40 ± 0.51	36.50 ± 0.84	155.21 ± 1.23	122.25 ± 1.44	28.09 ± 2.27	0.92 ± 0.03	CC
6	7.93 ± 0.25	31.17 ± 0.30	107.55 ± 1.43	94.09 ± 1.98	12.74 ± 0.18	2.90 ± 0.06	CC
7	15.87 ± 0.40	12.25 ± 0.14	44.01 ± 0.27	38.49 ± 0.67	5.89 ± 0.20	2.36 ± 0.02	CC
8	13.56 ± 0.27	22.73 ± 0.71	74.01 ± 0.80	53.60 ± 0.90	19.30 ± 0.38	9.84 ± 0.16	CC
9	7.71 ± 0.10	14.28 ± 0.22	34.96 ± 0.50	25.35 ± 0.55	6.76 ± 0.16	1.38 ± 0.03	CC
10	9.95 ± 0.14	15.35 ± 0.33	65.63 ± 0.92	55.30 ± 0.43	12.44 ± 0.20	1.96 ± 0.04	CC
11	3.75 ± 0.09	11.42 ± 0.30	27.58 ± 0.27	17.32 ± 0.21	7.45 ± 0.11	1.67 ± 0.04	CC
12	11.49 ± 0.20	14.44 ± 0.22	48.25 ± 0.29	41.82 ± 0.38	6.15 ± 0.15	4.54 ± 0.08	GBC
13	18.14 ± 0.11	34.87 ± 0.32	129.35 ± 060	100.05 ± 0.85	27.68 ± 0.31	8.80 ± 0.16	GBC
14	2.72 ± 0.04	8.34 ± 0.12	46.73 ± 0.42	34.92 ± 0.34	7.15 ± 0.08	3.36 ± 0.04	GBC
15	6.30 ± 0.21	7.92 ± 0.10	58.99 ± 0.49	44.81 ± 0.58	11.23 ± 0.23	1.08 ± 0.03	GBC
16	0.38 ± 0.01	0.98 ± 0.04	ND	ND	ND	1.77 ± 0.04	Normal
17	5.37 ± 0.09	21.32 ± 0.19	111.71 ± 0.46	99.23 ± 1.09	11.88 ± 0.20	3.50 ± 0.04	Normal
18	7.02 ± 0.08	10.85 ± 0.41	112.04 ± 0.92	82.25 ± 1.21	24.78 ± 0.31	1.87 ± 0.05	Normal
19	1.41 ± 0.02	2.86 ± 0.10	10.03 ± 0.22	4.68 ± 0.10	5.12 ± 0.13	2.13 ± 0.03	Normal

[a]The concentrations are expressed in mM. The quantity for each component is given in (mean ± standard deviation) derived from five independent integral measurements. ND, Could not be detected; CC, chronic cholecystitis; XGC, xanthogranulomatous cholecystitis; GBC, gallbladder cancer; Normal, no diagnosed gallstone disease.

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Single-Step Analysis of Individual Conjugated Bile Acids in Human Bile Using ^{1}H NMR Spectroscopy

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ABSTRACT: ^{1}H and ^{13}C NMR spectra of intact human bile were assigned using one-dimensional (^{1}H and ^{13}C) and two-dimensional (^{1}H-^{1}H and ^{1}H-^{13}C) experiments. Individual conjugated bile acids—glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, and taurochenodeoxycholic acid—were identified. The bile acids were quantified accurately and individually in a single step by using distinct and characteristic amide signals. Making use of ^{13}C NMR, the study also suggests a way to analyze unconjugated bile acids separately, if present. Chemical shift assignments and rapid single-step analysis of individual conjugated bile acids from intact bile presented herein may have immense utility in the study of bile acid metabolism and deeper understanding of hepatobiliary diseases.

Paper no. L9954 in *Lipids 41*, 591–603 (June 2006).

Bile acids have been implicated in the digestion and absorption of fats and in several other important physiological functions, including cholesterol homeostasis and bile flow, help in the excretion or recirculation of drugs, vitamins, and endogenous and exogenous toxins (1,2). Recently, new biological properties of bile acids as signaling molecules and as regulators of their own synthesis have also been reported (3). Abnormal composition of the conjugated bile salts in bile may lead to various hepatobiliary and gastrointestinal diseases (4–6). Dietary fat malabsorption and gallstone formation are the common causes of abnormal bile composition in the bile (4,6). Conditions that prevent the synthesis of bile acids or their excretion cause cholestasis or impaired bile flow. Several human diseases arising from altered levels of bile acids underscore the essential role of bile acids in metabolism (7). Bile acids are considered to be involved in the development of biliary tract carcinoma, although the underlying mechanisms are yet to be established (8). They have long been implicated in the etiology of colorectal cancer, but epidemiologic evidence remains elusive (9). Deoxycholic acid is reported to cause higher risk for pressure ulcers in elderly bedridden patients (10). It has been suggested that the bile acids in the feces act as promoters of colon cancer, and among the bile acids, deoxycholic acid is said to have strong influence. The ratio of deoxycholic acid to cholic acid in feces is reported to have a diagnostic significance in colon cancer (11). Several reports indicate that bile acids cause DNA damage and act as carcinogens (12). Aggravation of taurochenodeoxycholic acid induced liver damage, and hepatocyte apoptosis is reported due to the inhibition of phosphatidylinositol 3-kinase (13). From the experiments with immortalized mouse cholangiocytes using several bile acids, carcinogenic potential of glycochenodeoxycholic acid in biliary tract has been demonstrated (8). Bile acid synthetic defects that are uncommon genetic disorders were reported to be responsible for approximately 2% of persistent cholestasis in infants. These defects lead to liver diseases that may be life threatening but are treatable by replacement of deficient primary bile acids (14).

In view of the multitude of useful as well as deleterious roles played by the bile acids, measurement of their conjugation pattern is necessary for understanding the pathophysiology of these diseases. Several methods for bile acids analysis have been proposed (15–25). Of these, liquid chromatography–mass spectrometry (22–25) and liquid chromatography–electrospray tandem mass spectrometry (2) methods are reported to be useful for routine qualitative/quantitative analysis. These methods are not straightforward since they involve tedious steps such as extraction, hydrolysis, derivatization, and/or purification before analyses. A simple and convenient method for determining identity and quantity of the individual bile acids has not been reported. Nuclear magnetic resonance (NMR) spectroscopy offers immense potential in this direction. However, major difficulties of NMR for such applications arise from severe overlap of signals of a large number of bile components. As a part of our efforts to unravel the signals from complex bile spectra for single-step analysis of several bile components, we reported ^{1}H and ^{13}C chemical shifts, under physiological conditions, for several conjugated and unconjugated bile acids that are supposedly found in bile/gastrointestinal fluids (26) and, subsequently, identified conjugated bile acid marker signals in human bile (27). In continuation, we present here assignments, identification, and

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Abbreviations: DQF-COSY, double quantum filtered correlated spectroscopy; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; NOE, nuclear Overhauser enhancement; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TOCSY, total correlated spectroscopy; TSP, sodium salt of trimethylsilylpropionic acid-d_4.

single-step analysis of all major conjugated bile acids in human bile individually using simple ^{1}H NMR experiments.

EXPERIMENTAL PROCEDURE

Chemicals and human bile specimens. Sodium salts of glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), deuterium oxide (D_2O), and sodium salt of trimethylsilylpropionic acid-d_4 (TSP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Discarded specimens of human gallbladder bile from patients undergoing cholecystectomy (laparoscopic or open) for symptomatic gallstone disease were used for the study.

NMR experiments. All ^{1}H and ^{13}C NMR experiments of bile specimens were performed on Bruker Biospin Avance 400-, 700-, and/or 800-MHz NMR spectrometers using a 5-mm broadband or broadband inverse probe head (400 MHz) or cryoprobe heads (700 and 800 MHz), all equipped with shielded *z*-gradient accessories. Unless otherwise mentioned, all bile samples were prepared by diluting gallbladder bile by a factor of 10 using distilled water and NMR experiments were performed by using similar parameters as mentioned in our previous article in this issue.

One-dimensional (1D) ^{1}H NMR experiments. The 1D ^{1}H NMR experiments were performed with and/or without homonuclear decoupling (of glycine and taurine CH_2 protons of conjugated bile acids) by using a single-pulse sequence with suppression of water signal by presaturation during relaxation delay of 6 s. For homonuclear decoupled spectra, Gausssian multiplication window function was used with line and Gaussian broadening of –1.0 and 0.1 Hz, respectively.

1D ^{13}C NMR experiments. The 1D ^{13}C spectra were obtained by using one-pulse sequence, spin echo Fourier transform (SEFT), and quaternary carbon detection (QCD) (28) sequences with proton decoupling using WALTZ-16 composite pulse sequence. Typical parameters used were as follows: spectral width: 24,000, 42,000, and 48,000 Hz (at 400-, 700-, and 800-MHz spectrometers, respectively); data points: 32 K; acquisition time: 0.68, 0.39, and 0.34 s (at 400, 700, and 800 MHz, respectively); recycle delay: 3 s; number of transients: 2,000; spectrum size: 32 K points; and line broadening for exponential data multiplication: 3 Hz.

Assignment of ^{1}H and ^{13}C NMR spectra. Assignments of ^{1}H and ^{13}C signals of bile spectra were made by using the combination of 1D and 2D spectra. The signals in the 2D spectra were broad and weak due to the short transverse relaxation times of the signals arising from the aggregation of major bile components. The library of ^{1}H and ^{13}C chemical shifts (26), partial assignments on bile in aqueous media (27), and the assignments of bile dispersed in a polar organic solvent aided identification of signals in the complex spectra and distinguishing bile acids signals from lipids and cholesterol

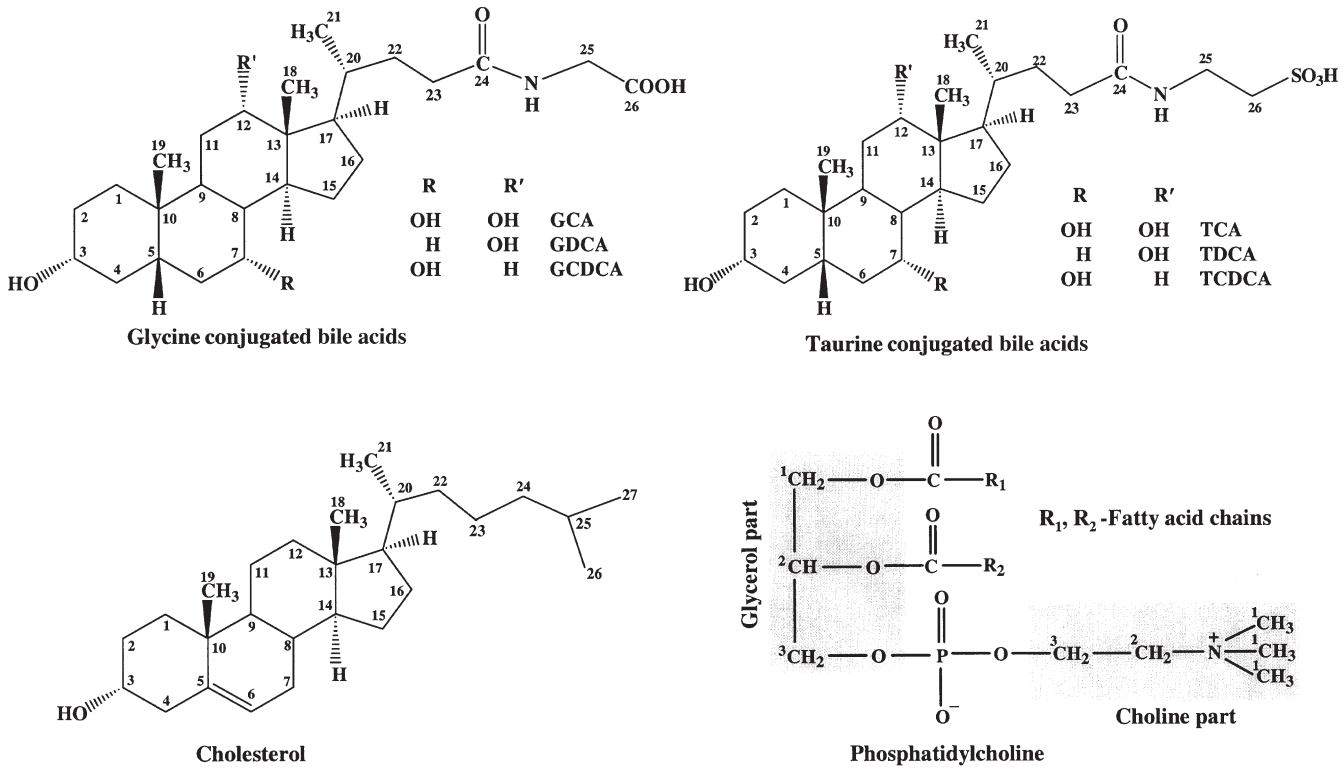

FIG. 1. Structures and numbering of protons and carbons of bile components identified in bile. The numbering of the proton(s) is the same as that of the corresponding directly attached carbons. The orientations of some substitutions in cholesterol and bile acids are marked with respect to the plane of the ring (cross-hatched) wedge: α substitution; bold wedge: β substitution).

signals (L9947 i.e. 29). Assignment from the combination of double quantum filtered correlated spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY), and multiplicity edited heteronuclear single quantum correlation; (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra at 700 and 800 MHz confirmed assignments made at 400 MHz.

Identification of individual conjugated bile acids. In one-dimensional ^{1}H spectra, broad amide signals from conjugated bile acids, which invariably appear in the isolated region

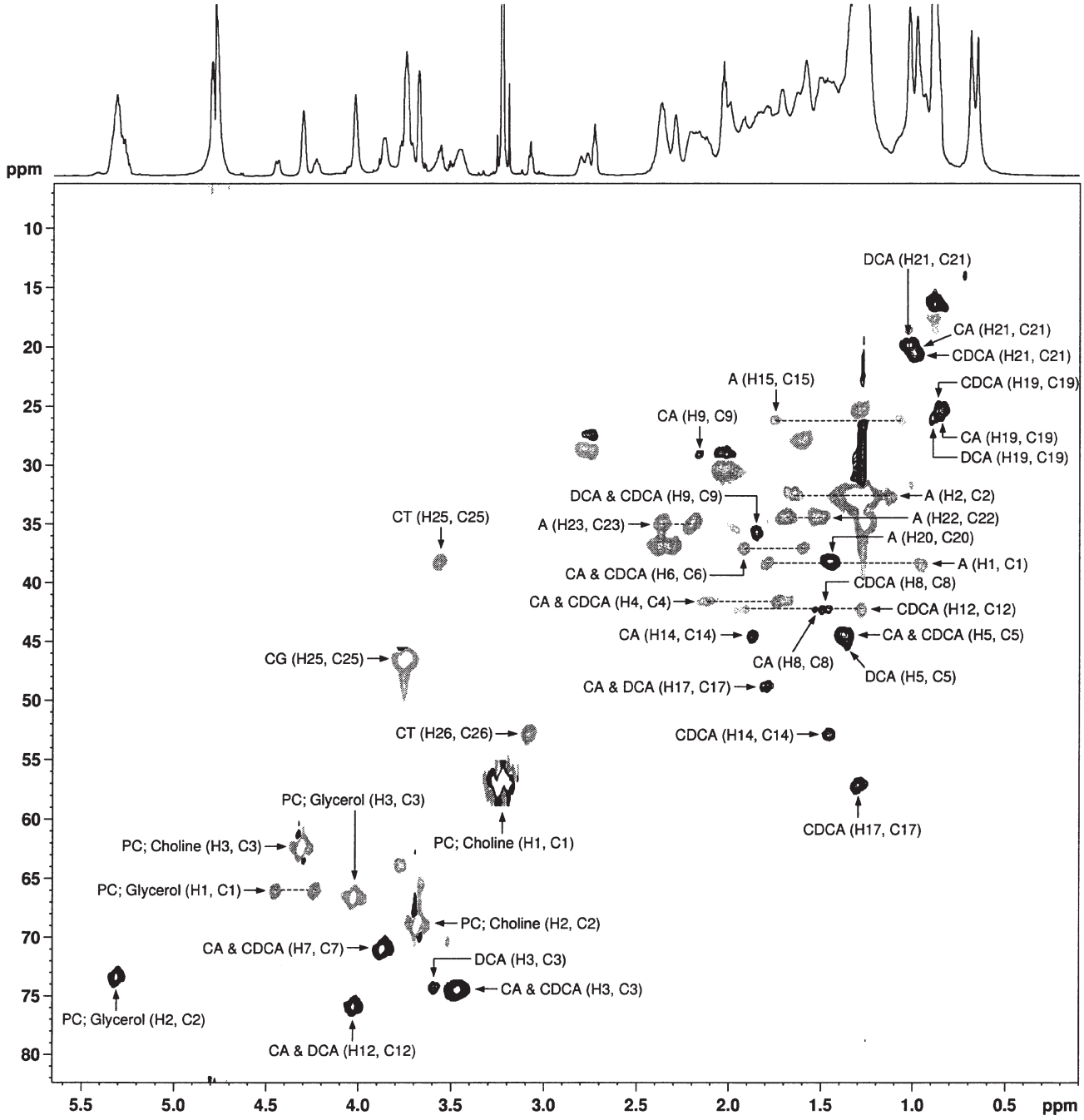

FIG. 2. Sensitivity-enhanced multiplicity edited ^{1}H-^{13}C HSQC spectrum of typical bile (50 µL diluted to 500 µL with water) obtained on a Bruker Biospin Avance 700-MHz spectrometer, along with the assignments of the cross peaks of protons and the attached carbons. For clarity, CH and CH_3 cross peaks are shown as dark continuous contours whereas CH_2 cross peaks are shown in light discontinuous contours. Wherever the chemical shifts of methylene protons (CH_2) are different (nondegenerate), two distinct cross peaks were observed corresponding to H_α and H_β protons (each pair of cross peaks is joined by dashed lines). PC, phosphatidylcholine; CT, conjugated taurine; CG, conjugated glycine; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxycholic acid. A: All glycine/taurine conjugated bile acids. The numbering of protons/carbons is as shown for the structures in Figure 1. A trace of 1D ^{1}H spectrum is shown on the 2D spectrum.

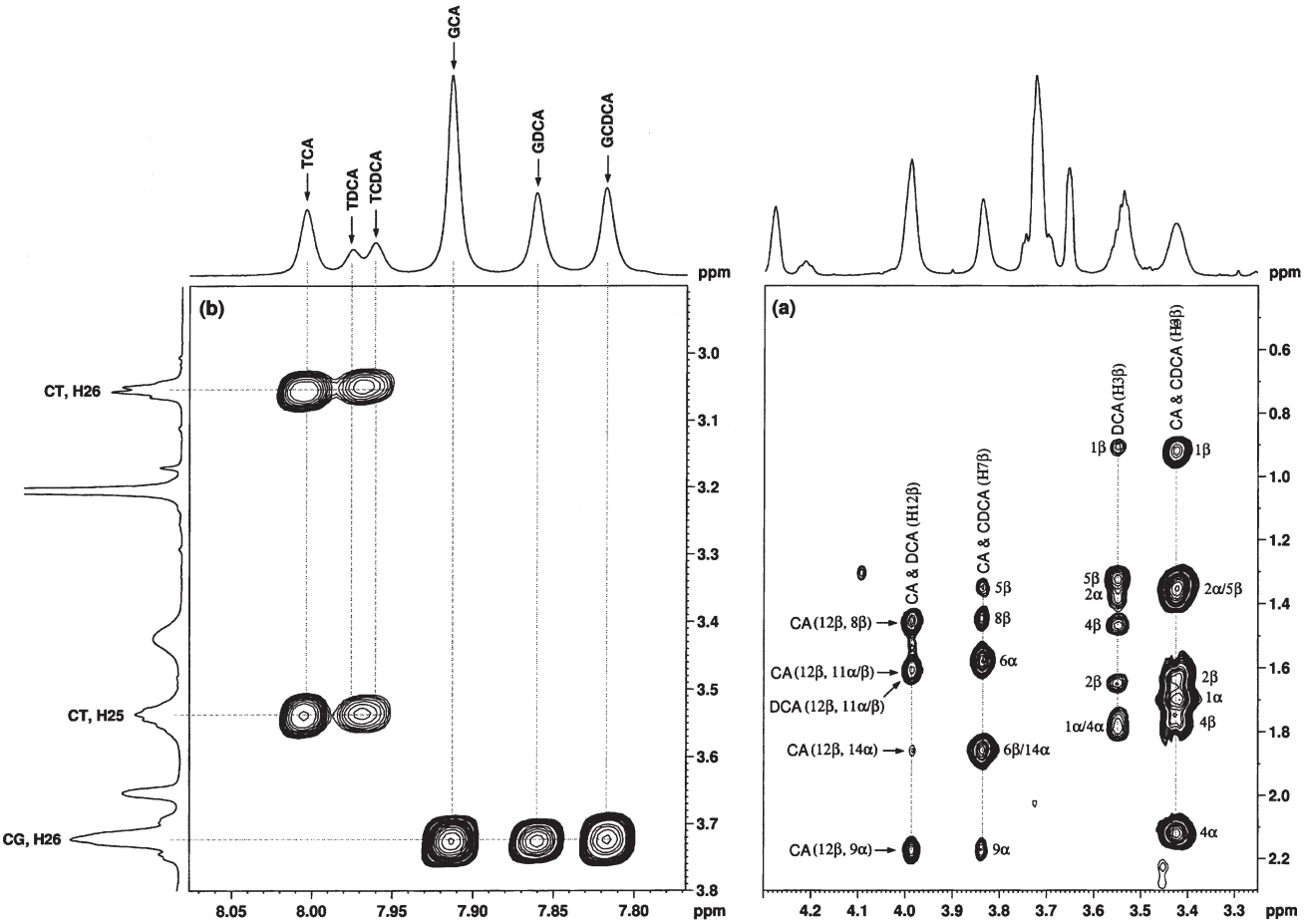

FIG. 3. Parts of TOCSY spectrum of typical bile (50 µL diluted to 500 µL with water) obtained on a Bruker Biospin AVANCE 800-MHz spectrometer. CT, conjugated taurine; CG, conjugated glycine; GCA, glycocholic acid; GDCA, glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxy cholic acid. The numbering of protons is as shown in Figure 1. A trace of 1D ^{1}H spectrum is shown on the 2D spectrum.

(7.77 to 8.03 ppm) of the bile spectra, were nearly overlapping with each other. Experiments were performed to improve the resolution so as to identify the individual conjugated bile acids. The one-dimensional ^{1}H experiments were performed by using homonuclear decoupling sequence with decoupler offset set at 3.59 ppm, which is in between the chemical shifts of methylene protons (25-CH_2) of conjugated glycine (3.75 ppm) and conjugated taurine (3.56 ppm). From the analysis of the combination of spectra from 1D decoupled DQF-COSY and TOCSY experiments, two groups of amide signals were identified. Each group had three signals, one group corresponded to glycine-conjugated bile acids, and the other corresponded to taurine-conjugated bile acids. By comparison of these resolved amide and other ^{1}H and ^{13}C signals of bile acids with the library of chemical shifts of standard conjugated bile acids (26), the three amide signals of glycine were assigned to GCDCA (7.84 ppm), GDCA (7.88 ppm), and GCA (7.93 ppm) and the three amide signals of taurine-conjugated bile acids were assigned to TCDCA (7.98 ppm), TDCA (8.00 ppm), and TCA (8.02 ppm). To further confirm the assignments of the amide signals, ^{1}H spectra of bile were recorded separately at 800 MHz with simultaneously decoupling both 25-CH_2 signals of conjugated glycine and taurine, before and after the addition of each standard conjugated bile acid.

Bile acids recovery test. After identifying the six bile acids individually (GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA) through their characteristic amide marker signals, NMR experiments were performed to test the accuracy and precision of quantitative analysis for each bile acid. The pH of a bile sample (4.0 mL diluted to 9.0 mL) was brought down (27) in the range 6 ± 0.5 using 6 N HCl, and, using this solution, six duplicate set of solutions (each 500 µL) were prepared in 5-mm NMR tubes. A reusable coaxial capillary containing quantitative reference, TSP (dissolved in 35 µL of D_2O), was inserted into the tube and ^{1}H spectra were recorded at 800 MHz by decoupling 25-CH_2 protons of conjugated glycine and taurine. The quantity of TSP in the capillary was

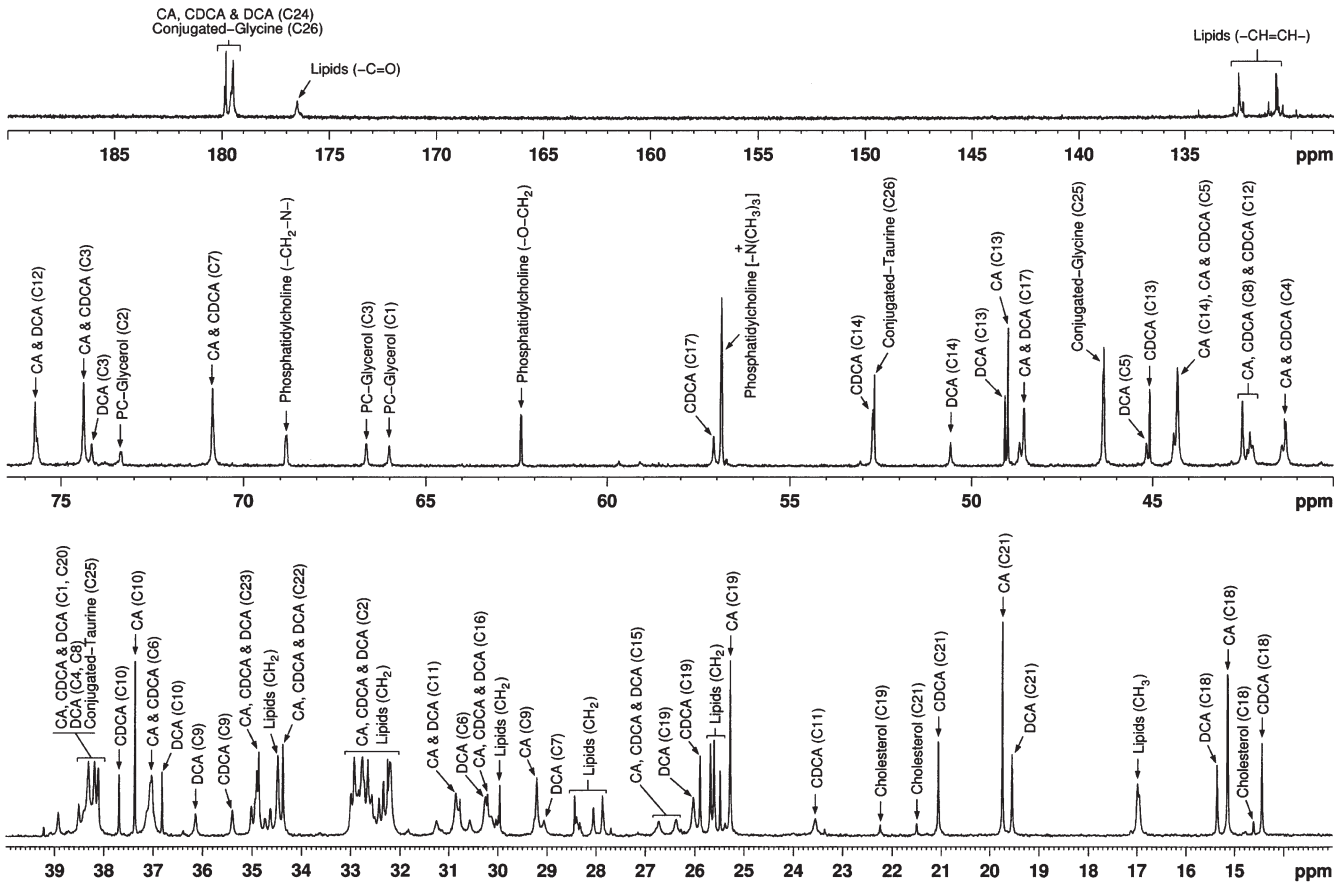

FIG. 4. Parts of ^{13}C spectrum of typical bile obtained on a Bruker Biospin Avance 800-MHz spectrometer with the assignments of most of the signals. PC, phosphatidylcholine; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxycholic acid. The numbering of protons/carbons is as shown for the structures in Figure 1.

precalibrated by ^1H NMR experiment against a signal from a known concentration of a standard sample (30). Integral areas of the amide signals of all the six conjugated bile acids were determined relative to TSP by deconvolution of the signals using Xwinnmr software version 3.5 (Bruker Biospin Fällanden, Switzerland). Subsequently, known quantities of standard bile acids, GCA (1.04 mg), GDCA (0.84 mg), GCDCA (0.87 mg), TCA (0.87 mg), TDCA (0.84 mg), or TCDCA (0.75 mg), were added to the diluted bile solutions taken in separate sets of NMR tubes. ^1H NMR spectra were recorded again, under identical conditions, and the integrals of the amide signals were determined from the resultant spectra relative to the TSP signal. The quantity of each standard conjugated bile acid recovered through NMR was calculated from the corresponding amide integral (difference of amide integral before and after the addition of bile acid) and compared with the actual quantity of the added bile acid. In each case, deconvolution of the amide signals was repeated five times independently and the mean quantity of recovered bile acid along with the standard deviation was determined.

Quantitative estimation of bile acids. To demonstrate single-step analysis of individual conjugated bile acids, NMR experiments were performed on gallbladder bile specimens from 16 patients. The pH of each bile specimen was brought down in the range 6 ± 0.5 by addition of 1–2 μL of 6 N HCl. A reusable coaxial capillary tube containing TSP in D_2O was inserted into the NMR tube before recording the spectra. ^1H spectra at 800 MHz were obtained with decoupling 25-CH_2 protons of conjugated glycine and taurine. Integral areas of all the six amide signals were determined relative to TSP by deconvolution. Subsequently, quantities of all the six bile acids were determined, individually, using a computer program that takes into account integral area and molecular weight of each conjugated bile acid and the reference (TSP). In each case, deconvolution of the amide signals was repeated five times independently and the mean quantities along with the standard deviation were determined for each bile acid.

To determine whether decoupling of glycine and taurine methylene protons (25-CH_2) would alter the intensities of amide protons due to nuclear Overhauser enhancement (NOE) (if any), one-dimensional ^1H NMR experiments were performed on one of the bile specimens by suppressing the water signal using presaturation with 2-s acquisition time (during which homonuclear decoupling was also performed) and 45° excitation pulse. Spectra were recorded with and without decoupling of methylene protons by varying the re-

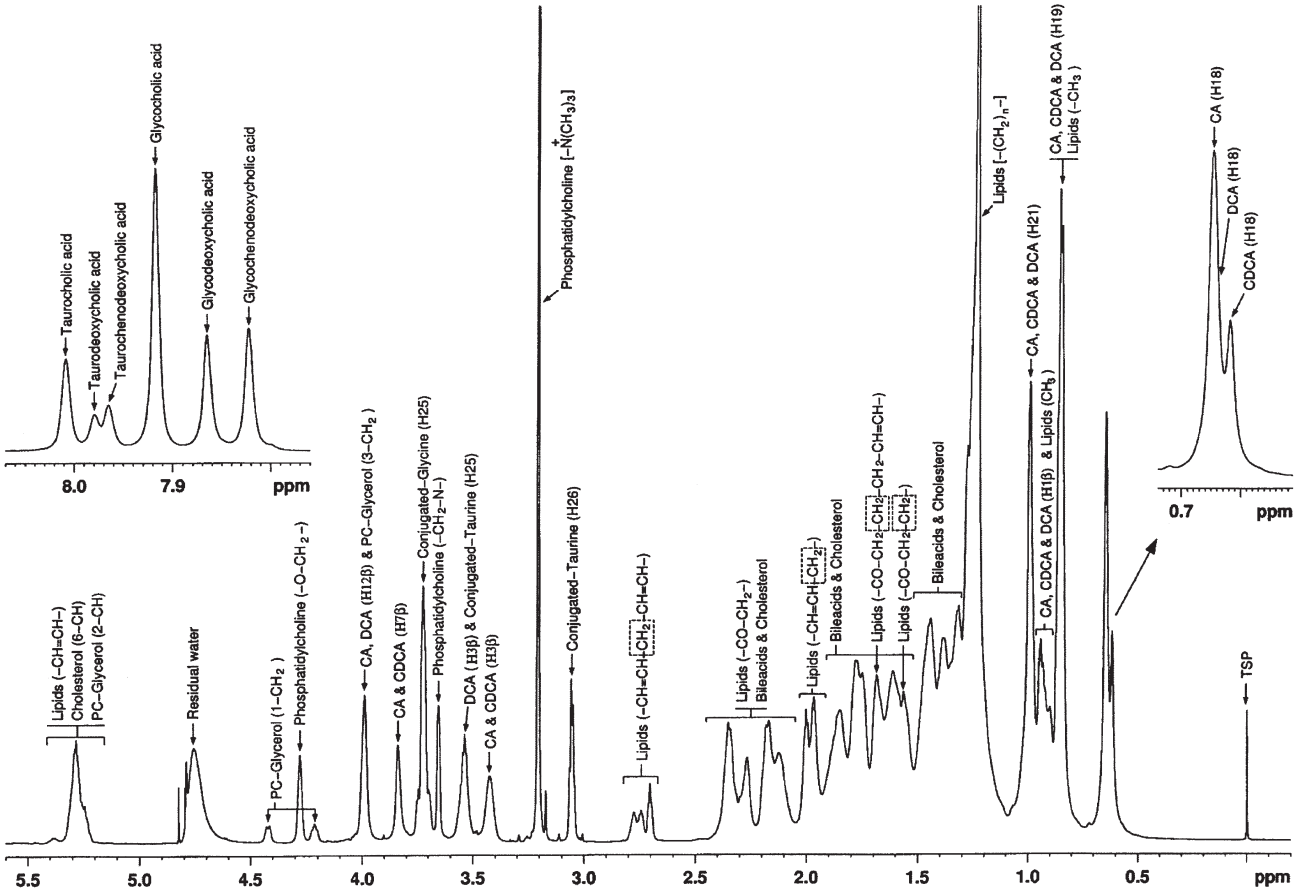

FIG. 5. Parts of ^{1}H spectrum of typical bile obtained on a Bruker Biospin Avance 800-MHz spectrometer with the assignments of most of the signals. PC, phosphatidylcholine; CA, glycine/taurine-conjugated cholic acid; DCA, glycine/taurine-conjugated deoxycholic acid; CDCA, glycine/taurine-conjugated chenodeoxycholic acid. The numbering of protons/carbons is as shown for the structures in Figure 1. Inset shows six amide signals, each corresponding to conjugated bile acid as indicated, obtained after decoupling 25-CH_2 protons of conjugated glycine and taurine.

cycle delay from 1 to 10 s in steps of 1 s. The integrals of all the amide signals at different recycle delay were obtained and compared.

RESULTS

Figure 1 shows the structures of bile components that are identified in intact gallbladder bile. Bile specimens from different patients were qualitatively consistent in terms of the signal pattern, but greatly varied in intensities of the signals from individual bile components. The cross peaks, particularly in heteronuclear 2D spectra, were weak in intensity due to short transverse relaxation times of the signals arising from aggregation of the bile components under natural conditions. Signals from cholesterol, lipids, and bile acids were identified from the analysis of two-dimensional spectra from DQF-COSY, TOCSY, multiplicity edited HSQC and HMBC experiments at 400, 700, and 800 MHz. Due to the structural similarity of various conjugated bile acids, most of the ^{1}H and ^{13}C signals of bile acids overlap, as shown in the typical edited HSQC and TOCSY spectra in Figures 2 and 3, respectively. Distinct chemical shifts of carbons C9, C10, C13, C18, C19, C21 of CA; C3, C5, C9, C10, C13, C14, C18, C19, C21 of DCA; and C9, C10, C11, C13, C17, C18, C19, C21 of CDCA, as shown in Figure 4, arising from the substitution effect (26) at position 7 and/or 12 (Fig. 1) enabled clear identification of CA, DCA, and CDCA moieties of the conjugated bile acids. Influence of glycine or taurine conjugation on almost all $^{1}H/^{13}C$ chemical shifts of bile acid moieties was minimal except in the vicinity of conjugation (Figs. 4 and 5). However, amide signals were distinctly observed for each conjugated bile acid. These amide signals appear very close to each other (7.77–8.03 ppm) at low magnetic fields (400 MHz) (Fig. 6a). However, at higher magnetic fields (700/800 MHz), four of six signals were resolved (Fig. 6d). Each amide signal is a triplet arising from indirect spin-spin coupling between amide (NH) and 25-CH_2 protons of conjugated glycine or taurine (Fig. 1). Upon removal of indirect spin-spin couplings by simultaneously decoupling the methylene protons of conjugated glycine (3.75 ppm) and conjugated taurine (3.56 ppm), amide signals further get resolved and all six bile acids signals were separated at 700 and 800 MHz (Fig. 6e). At 400 MHz, amide signals of TCDCA and TDCA were still overlapping (Fig. 6b).

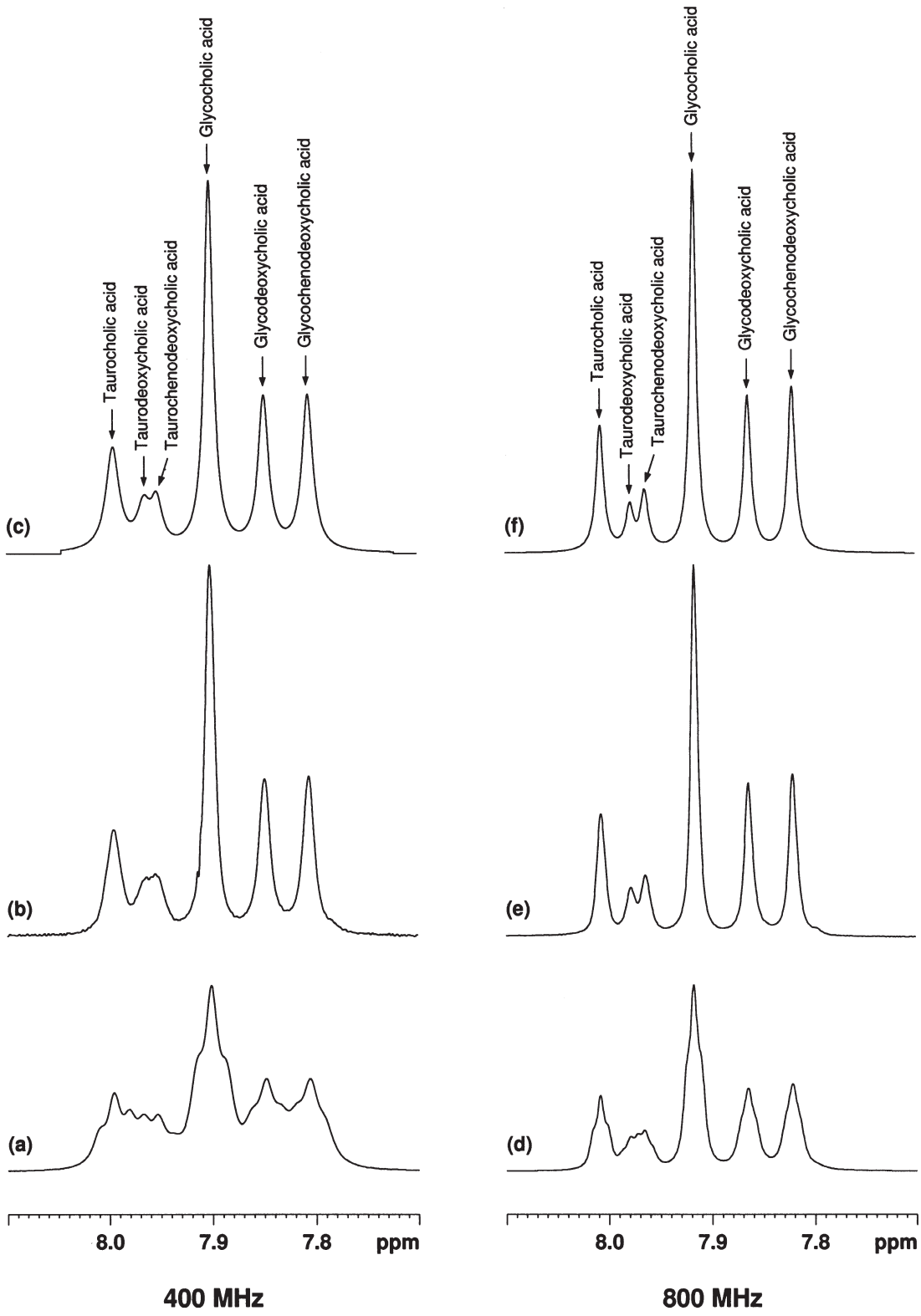

FIG. 6. Amide regions of bile spectra showing characteristic signals of glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and taurocholic acid (TCA). The spectra were obtained at 400 and 800 MHz without (a and d) and with (b and e) decoupling of 25-CH_2 protons of conjugated glycine and taurine. At 400 MHz, two of the amide signals (taurochenodeoxycholic acid and taurodeoxycholic acid) are still overlapping (b). All six amide signals are distinctly visible in the spectrum recorded at 800 MHz (d). The regions shown in c and f are the deconvoluted (Lorentzian) decoupled spectra b and e, respectively.

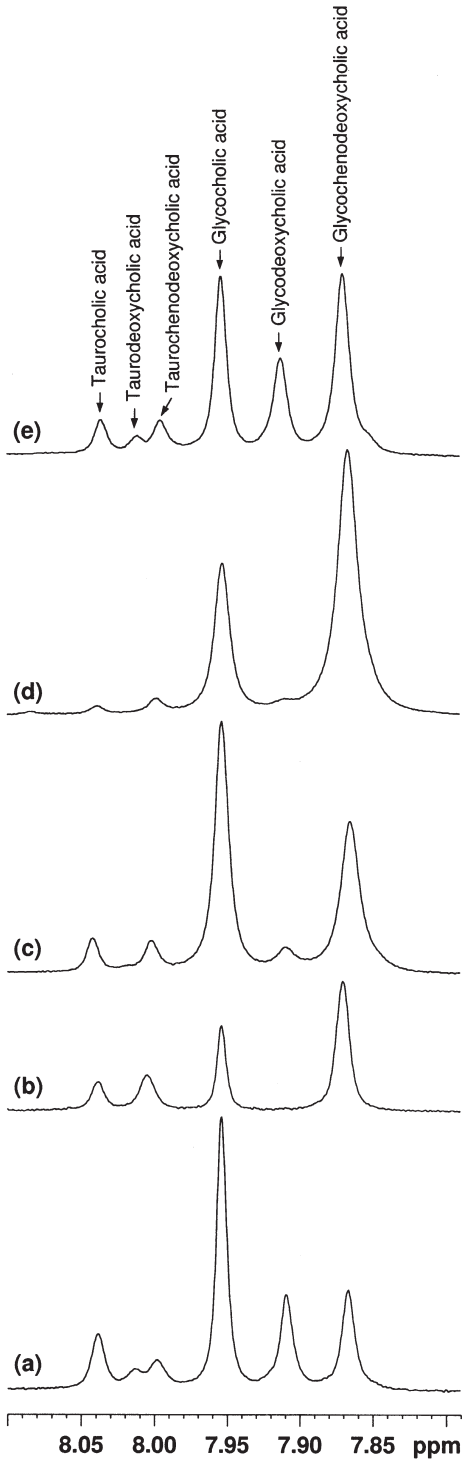

FIG. 7. Parts of 800-MHz ^{1}H NMR spectra of gallbladder bile (50 µL diluted to 500 µL with water) from five typical patients with gallbladder disease highlighting the variation of the relative intensities of the bile acids within as well as between the bile specimens (see also Table 4). All the spectra were plotted under identical conditions for direct comparison of the relative quantities of various bile acids. The bile samples were from patients (a) with chronic cholecystitis (CC); (b) with xanthogranulomatous cholecystitis (XGC); (c) normal (with no diagnosed gallstone disease); (d) with xanthogranulomatous cholecystitis (XGC); and (e) with chronic cholecystitis (CC).

Experiments performed before and after the addition of authentic conjugated bile acids confirmed the assignment of individual conjugated bile acids in bile. The ^{1}H and ^{13}C chemical shifts for the glycine- and taurine-conjugated bile acids in bile measured using 1D and 2D spectra are shown in Tables 1 and 2.

A typical best fit spectrum obtained after deconvolution of the decoupled amide region of a bile spectrum (Fig. 6e) is shown in Figure 6f. Integrals of individual amide signals in the best fit spectra were used for determining absolute quantities of individual conjugated bile acids. As shown in Table 3, the bile acids thus determined showed excellent agreement between the quantity of bile acid added and that recovered using ^{1}H NMR. Rapid and single-step analyses of individual conjugated bile acids were demonstrated on 16 human gallbladder bile specimens and the quantities of six bile acids thus determined are shown in Table 4. Significant differences in the quantities of bile acids within the bile and among various bile specimens were observed. Typical spectra for five gallbladder bile specimens, highlighting the variation in the absolute and relative quantities of individual bile acids in each bile specimen, are shown in Figure 7. Experiments performed to determine if there is any NOE buildup on the amide protons showed no change in integral of the amide protons in any of the spectra obtained with or without decoupling of the methylene protons of conjugated glycine and taurine.

DISCUSSION

Although NMR spectroscopy has tremendous advantages over conventional analytical methods, until today, its applications to human bile has been limited due to the complexity of the spectra arising from aggregation of major bile components such as cholesterol, phospholipids, and bile acids, which lead to broad signals. Several bile acids that are reported to be a part of major components of bile have very close structural resemblance, resulting in the severe overlap of most of the bile acid signals with each other, thus making the spectra further complicated to analyze. In the present study, assignment of ^{1}H and ^{13}C spectra of intact bile was greatly aided by the following sequential studies, which were aimed at devising a simple single-step method for simultaneously analyzing a large number of bile components that are detected by NMR: (a) establishment of the library of ^{1}H and ^{13}C chemical shifts by using various 1D and 2D experiments for several conjugated and unconjugated bile acids which are thought to be the components of human bile (26); (b) identification of bile acids and their distinct marker signals in human bile by using 1D and 2D experiments with the knowledge of the accurate chemical shifts of standard bile acids; in this study, amide signals were identified in human bile as conjugated bile acids markers (27); (c) assignment of ^{1}H and ^{13}C spectra of intact bile by using NMR experiments at 400 and 700 MHz after dispersing the bile in a polar organic solvent, which drastically reduced the signal line width due to increased transverse relaxation times and improved the spectral resolution (29).

TABLE 1
^{1}H and ^{13}C Chemical Shifts of Glycocholic Acid, Glycochenodeoxycholic Acid, and Glycodeoxycholic Acid from Intact Human Gallbladder Bile[a]

		Glycocholic acid			Glycochenodeoxycholic acid			Glycodeoxycholic acid		
			Proton			Proton			Proton	
No.	Type	Carbon	α	β	Carbon	α	β	Carbon	α	β
1	CH_2	38.30	1.80	0.96	38.30	1.80	0.96	38.30	1.80	0.96
		(37.66)	(1.80)	(1.00)	(38.10)	(1.83)	(0.96)	(38.17)	(1.81)	(0.98)
2	CH_2	32.24	1.38	1.65	32.24	1.38	1.65	32.24	1.40	1.65
		(32.06)	(1.39)	(1.66)	(32.49)	(1.38)	(1.64)	(32.02)	(1.42)	(1.69)
3	CH	74.38	—	3.46	74.38	—	3.46	74.15	—	3.58
		(74.43)		(3.49)	(74.41)		(3.47)	(74.21)		(3.63)
4	CH_2	41.31	2.12	1.70	41.31	2.12	1.70	38.18	1.81	1.53
		(41.20)	(2.07)	(1.70)	(41.27)	(2.08)	(1.69)	(37.98)	(1.81)	(1.54)
5	CH	44.31	—-	1.38	44.39	—	1.38	45.17	—	1.36
		(43.90)		(1.44)	(44.12)		(1.40)	(44.93)		(1.44)
6	CH_2	37.03	1.60	1.91	37.03	1.60	1.91	30.25	1.35	1.84
		(36.68)	(1.55)	(1.97)	(36.98)	(1.56)	(1.96)	(30.00)	(1.34)	(1.85)
7	CH	70.85	—	3.87	70.85	—	3.87	29.06	1.39	1.23
		(71.13)		(3.89)	(70.99)		(3.87)	(28.99)	(1.45)	(1.23)
8	CH	42.51	—	1.51	42.51	—	1.43	38.52	—	1.37
		(42.06)		(1.59)	(42.13)		(1.45)	(38.87)		(1.44)
9	CH	29.21	2.16	—	35.40	1.84	—	36.14	1.84	—
		(29.18)	(2.12)		(35.32)	(1.81)		(36.22)	(1.85)	
10	C	37.36	—	—	37.69	—	—	36.82	—	—
		(37.18)			(37.63)			(36.77)		
11	CH_2	30.85	1.58	1.58	23.55	1.52	1.27	30.85	1.58	1.58
		(30.54)	(1.59)	(1.59)	(23.46)	(1.50)	(1.26)	(31.14)	(1.55)	(1.55)
12	CH	75.72	—	4.03	42.30	1.26	1.97	75.72	—	4.03
		(75.94)		(4.05)	(42.18)	(1.25)	(1.98)	(75.85)		(4.05)
13	C	48.99	—	—	45.08	—		49.08	—	—
		(48.95)			(45.14)			(49.06)		
14	CH	44.31	1.87	—	52.72	1.45	—	50.58	1.58	—
		(44.36)	(1.83)		(52.76)	(1.42)		(50.60)	(1.61)	
15	CH_2	26.38	1.74	1.07	26.38	1.74	1.07	26.72	1.74	1.07
		(25.73)	(1.71)	(1.12)	(26.30)	(1.71)	(1.07)	(26.60)	(1.67)	(1.07)
16	CH_2	30.20	2.01	1.35	30.20	2.01	1.35	30.20	2.01	1.35
		(30.04)	(1.96)	(1.29)	(30.71)	(1.96)	(1.27)	(30.42)	(1.95)	(1.27)
17	CH	48.56	1.78	—	57.09	1.29	—	48.56	1.78	—
		(49.21)	(1.74)		(57.68)	(1.25)		(49.13)	(1.77)	
18	CH_3	15.14	0.69		14.44	0.65		15.36	0.68	
		(14.91)	(0.71)		(14.45)	(0.67)		(15.42)	(0.72)	
19	CH_3	25.28	0.84		25.90	0.87		26.04	0.89	
		(24.90)	(0.91)		(25.57)	(0.92)		(25.83)	(0.94)	
20	CH	38.29	1.45		38.29	1.45		38.29	1.45	
		(37.95)	(1.44)		(38.16)	(1.46)		(38.36)	(1.44)	
21	CH_3	19.73	1.02		21.05	0.98		19.54	1.02	
		(19.50)	(1.00)		(20.91)	(0.97)		(19.47)	(1.02)	
22	CH_2	34.38	1.49, 1.69		34.38	1.49, 1.69		34.38	1.49, 1.69	
		(34.37)	(1.43, 1.77)		(34.42)	(1.44, 1.72)		(34.37)	(1.46, 1.74)	
23	CH_2	34.86	2.37, 2.21		34.86	2.37, 2.21		34.86	2.37, 2.21	
		(35.29)	(2.36, 2.23)		(35.18)	(2.34, 2.17)		(35.33)	(2.39, 2.21)	
24	C	179.81	—		179.48	—		179.48	—	
		(180.02)			(179.64)			(179.67)		
25	CH_2	46.36	3.75		46.36	3.75		46.36	3.75	
		(46.13)	(3.74)		(46.20)	(3.73)		(46.19)	(3.75)	
26	C	179.59	—		179.59	—		179.59	—	
		(179.54)			(179.43)			(179.44)		
27	NH	—	7.93		—	7.84		—	7.88	
			(7.86)			(7.76)			(7.83)	

[a]The chemical shift values of the standard bile acids (26) are given in parentheses. Numbering for carbons/protons is as given in Figure 1.

All amide signals do not get resolved even at higher magnetic fields (Fig. 6d). This necessitated performing experiments with decoupling of methylene protons (25-CH_2) of glycine and taurine to obtain resolved signals. At lower magnetic fields (400 MHz), TDCA and TCDCA signals still overlap even after decoupling (Fig. 6b), whereas at higher mag-

TABLE 2
^{1}H and ^{13}C Chemical Shifts of Taurocholic Acid, Taurochenodeoxycholic Acid, and Taurodeoxycholic Acid from Intact Human Gallbladder Bile[a]

		Taurocholic acid			Taurochenodeoxycholic acid			Taurodeoxycholic acid		
			Proton			Proton			Proton	
No.	Type	Carbon	α	β	Carbon	α	β	Carbon	α	β
1	CH_2	38.30	1.80	0.96	38.30	1.80	0.96	38.30	1.80	0.96
		(37.74)	(1.80)	(0.99)	(38.09)	(1.83)	(1.00)	(38.20)	(1.80)	(0.97)
2	CH_2	32.24	1.38	1.65	32.24	1.38	1.65	32.24	1.40	1.65
		(32.06)	(1.38)	(1.65)	(32.48)	(1.37)	(1.66)	(32.02)	(1.40)	(1.67)
3	CH	74.38	—	3.46	74.38	—	3.46	74.15	—	3.58
		(74.39)		(3.47)	(74.46)		(3.48)	(74.24)		(3.62)
4	CH_2	41.31	2.12	1.70	41.31	2.12	1.70	38.18	1.81	1.53
		(41.19)	(2.08)	(1.70)	(41.30)	(2.08)	(1.70)	(38.12)	(1.80)	(1.52)
5	CH	44.31	—	1.38	44.31	—	1.38	45.17	—	1.36
		(43.95)		(1.43)	(44.10)		(1.41)	(44.93)		(1.42)
6	CH_2	37.03	1.60	1.91	37.03	1.60	1.91	30.25	1.35	1.84
		(36.74)	(1.55)	(1.94)	(36.96)	(1.57)	(1.96)	(30.01)	(1.31)	(1.84)
7	CH	70.85	—	3.87	70.85	—	3.87	29.06	1.39	1.23
		(71.04)		(3.87)	(71.07)		(3.87)	(28.99)	(1.42)	(1.22)
8	CH	42.51	—	1.51	42.51	—	1.43	38.52	—	1.37
		(42.16)		(1.55)	(42.11)		(1.48)	(38.89)		(1.42)
9	CH	29.21	2.16	—	35.40	1.84	—	36.14	1.84	—
		(29.18)	(2.14)		(35.32)	(1.82)		(36.24)	(1.82)	
10	C	37.36	—	—	37.69	—	—	36.82	—	—
		(37.22)			(37.64)			(36.78)		
11	CH_2	30.85	1.58	1.58	23.55	1.52	1.27	30.85	1.58	1.58
		(30.62)	(1.57)	(1.57)	(23.46)	(1.50)	(1.26)	(31.21)	(1.53)	(1.53)
12	CH	75.72	—	4.03	42.30	1.26	1.97	75.72	—	4.03
		(75.84)		(4.03)	(42.19)	(1.28)	(1.96)	(75.85)		(4.03)
13	C	48.99	—	—	45.08	—	—	49.08	—	—
		(48.96)			(45.17)			(49.07)		
14	CH	44.31	1.87	—	52.72	1.45	—	50.58	1.58	—
		(44.33)	(1.84)		(52.80)	(1.41)		(50.63)	(1.59)	
15	CH_2	26.38	1.74	1.07	26.38	1.74	1.07	26.72	1.74	1.07
		(25.78)	(1.72)	(1.09)	(26.29)	(1.72)	(1.08)	(26.60)	(1.65)	(1.05)
16	CH_2	30.20	2.01	1.35	30.20	2.01	1.35	30.20	2.01	1.35
		(30.09)	(1.95)	(1.26)	(30.73)	(1.97)	(1.27)	(30.42)	(1.92)	(1.22)
17	CH	48.56	1.78	—	57.09	1.29	—	48.56	1.78	—
		(49.10)	(1.74)		(57.71)	(1.24)		(49.16)	(1.75)	
18	CH_3	15.14	0.69		14.44	0.65		15.36	0.68	
		(14.99)	(0.69)		(14.43)	(0.67)		(15.42)	(0.70)	
19	CH_3	25.28	0.84		25.90	0.87		26.04	0.89	
		(24.99)	(0.90)		(25.54)	(0.92)		(25.84)	(0.92)	
20	CH	38.29	1.45		38.29	1.45		38.29	1.45	
		(37.96)	(1.41)		(38.13)	(1.44)		(38.33)	(1.42)	
21	CH_3	19.73	1.02		21.05	0.98		19.54	1.02	
		(19.51)	(0.99)		(20.91)	(0.96)		(19.51)	(0.99)	
22	CH_2	34.38	1.49, 1.69		34.38	1.49, 1.69		34.38	1.49, 1.69	
		(34.38)	(1.41, 1.73)		(34.45)	(1.43, 1.72)		(34.39)	(1.42, 1.71)	
23	CH_2	34.86	2.37, 2.21		34.38	2.37, 2.21		34.86	2.37, 2.21	
		(35.39)	(2.32, 2.18)		(35.27)	(2.31, 2.15)		(35.53)	(2.33, 2.16)	
24	C	179.81	—		179.48	—		179.48	—	
		(180.05)			(179.85)			(179.79)		
25	CH_2	38.11	3.56		38.11	3.56		38.11	3.56	
		(37.88)	(3.56)		(37.94)	(3.56)		(37.95)	(3.56)	
26	C	52.68	3.08		52.68	3.08		52.68	3.08	
		(52.69)	(3.07)		(52.70)	(3.07)		(52.69)	(3.07)	
27	NH	—	8.02		—	7.98		—	8.00	
			(7.96)			(7.93)			(7.92)	

[a]The chemical shift values of the standard bile acids (26) are given in parentheses. Numbering for carbons/protons is as given in Figure 1.

netic fields, all the signals get resolved (Fig. 6e). We have shown that decoupling of methylene protons of conjugated glycine and taurine does not cause any change in intensity of the amide protons and thus it rules out interference from the NOE buildup on the amide protons during decoupling of the adjacent methylene protons. In fact, with a 45° excitation

pulse, all the amides proton magnetization reaches equilibrium even at a recycle delay of 3 s (including acquisition time). Thus the relaxation times of the amide protons are favorable for rapid analysis of individual bile acids. The longer recycle delay of 6 s used in this study was, however, necessitated due to longer relaxation time of the reference signal (TSP) used for quantitative analysis.

It is clear from Tables 1 and 2 that most bile acid signals severely overlap with each other except amide proton signals. In our previous study, we identified an unresolved bunch of amide signals to be markers of total glycine- and taurine-conjugated bile acids in bile (27). In this study, from the rigorous analysis of bile spectra under natural conditions, we have unraveled them into six individual conjugated bile acids. In the whole bile ^{1}H NMR spectrum, amide signals are the only ones that are distinct signatures of individual conjugated bile acids. Under physiological conditions of bile (pH = 7.0 to 7.7), amide signal intensities are attenuated due to the chemical exchange and hence they do not represent true concentration of the bile acids. Earlier we have made a detailed study under variable pH conditions for human bile as well as for several standard conjugated bile acids and shown that the attenuation of amide signals arising from chemical exchange can be suppressed by reducing the pH to slightly lower than the physiological value (6.0 ± 0.5) (27). Therefore, quantitative analysis of individual bile acids was performed after the addition of 1–2 μL of hydrochloric acid to bile solutions so as to bring down the pH in the range of 6.0 ± 0.5 and, as expected, the quantities thus determined show good agreement with the known amounts in the standard addition experiments (Table 3). Alternatively, bile solution pH may be adjusted by the addition of aqueous buffer (pH = 6.0) instead of diluting the bile using distilled water. This may allow better control of the pH and hence the pH-dependent signal intensity variations, if any.

TABLE 3
Quantity of Bile Acids Recovered from Gallbladder Bile Through Increase in Amide Signal Integral in 1H NMR Versus the Quantity Added (Each Experiment Was Performed in Duplicate)[a]

Bile acid	Quantity added to bile (mg)	Quantity recovered by NMR (mg) (mean ± SD)	Error (%) Mean ± SD
Glycocholic acid	1.04	0.99 ± 0.003	4.77 ± 0.28
		1.00 ± 0.003	3.61 ± 0.28
Glycochenodeoxycholic acid	0.87	0.81± 0.01	3.95 ± 1.20
		0.81 ± 0.01	3.92 ± 1.04
Glycodeoxycholic acid	0.84	0.82 ± 0.002	1.73 ± 0.20
		0.82 ± 0.001	2.82 ± 0.14
Taurocholic acid	0.87	0.85 ± 0.001	2.01 ± 0.11
		0.86 ± 0.011	0.49 ± 1.27
Taurochenodeoxycholic acid	0.75	0.74 ± 0.007	0.78 ± 0.94
		0.77 ± 0.005	2.21 ± 0.66
Taurodeoxycholic acid	0.84	0.83 ± 0.003	1.75 ± 0.32
		0.87 ± 0..005	3.49 ± 0.54

[a]Mean and standard deviation for each recovered quantity of the bile acid were obtained from deconvolution of the amide signal five times, independently.

TABLE 4
Typical Concentrations of Six Conjugated Bile Acids Obtained Individually from Single-Step ^{1}H NMR Analysis in Gallbladder Bile of 16 Patients[a]

Serial Number	Glycocholic acid	Glycochenodeoxycholic acid	Glycodeoxy cholic acid	Taurocholic acid	Taurochenodeoxycholic acid	Taurodeoxycholic acid	Type of disease
1	3.97 ± 0	3.86 ± 0.01	0.82 ± 0.01	1.21 ± 0.01	1.05 ± 0.1	ND	CC
2	13.65 ± 0.08	7.89 ± 0.11	6.31 ± 0.05	3.28 ± 0.08	1.96 ± 0.05	1.07 ± 0.01	CC
3	18.76 ± 0.04	19.91 ± 0.09	5.83 ± 0.04	5.57 ± 0.06	5.74 ± 0.03	1.55 ± 0.01	CC
4	13.02 ± 0.03	9.29 ± 0.03	ND	0.58 ± 0.01	0.34 ± 0.01	ND	CC
5	17.23 ± 0.03	7.91 ± 0.08	7.11 ± 0.05	4.33 ± 0.05	2.29 ± 0.05	1.36 ± 0.01	CC
6	33.62 ± 0.24	23.18 ± 0.55	11.36 ± 0.19	6.05 ± 0.22	5.09 ± 0.23	1.59 ± 0.04	CC
7	18.36 ± 0.04	9.29 ± 0.02	3.06 ± 0.03	5.55 ± 0.01	2.71 ± 0	0.42 ± 0.01	CC
8	39.75 ± 0.19	30.57 ± 0.05	21.69 ± 0.12	6.52 ± 0.01	5.04 ± 0.16	3.37 ± 0.02	CC
9	29.05 ± 0.11	36.34 ± 0.06	17.67 ± 0.05	5.73 ± 0.05	6.27 ± 0.04	2.22 ± 0.01	CC
10	6.11 ± 0.03	6.79 ± 0.07	3.74 ± 0.01	1.36 ± 0.03	1.25 ± 0.02	0.59 ± 0.01	CC
11	28.16 ± 0.04	45.79 ± 0.22	ND	6.33 ± 0.14	9.47 ± 0.12	ND	CC
12	11.72 ± 0.01	15.36 ± 0.11	6.64 ± 0.02	2.40 ± 0.01	2.46 ± 0.01	1.21 ± 0.02	CC
13	62.65 ± 0.23	18.27 ± 0.11	8.80 ± 0.12	17.92 ± 0.09	5.36 ± 0.08	1.05 ± 0.03	Normal
14	28.39 ± 0.03	9.48 ± 0.06	10.36 ± 0.05	8.45 ± 0.05	4.08 ± 0.02	3.86 ± 0.01	CC
15	27.82 ± 0.06	22.56 ± 0.09	12.31 ± 0.03	4.08 ± 0.03	3.76 ± 0.03	1.60 ± 0.01	CC
16	19.56 ± 0.03	15.54 ± 0.14	7.74 ± 0.08	4.71 ± 0.09	4.88 ± 0.01	1.24 ± 0.01	CC

[a]Mean and standard deviation for each bile acid were obtained from deconvolution of the amide signal five times independently. ND = Not detected; CC = chronic cholecystitis (gallstone disease); Normal = no diagnosed gallstone disease.

As seen in Table 4, in all 16 bile specimens analyzed, three each of glycine- and taurine-conjugated bile acids (GCA, GDCA, GCDCA, TCA, TDCA, and TCDCA) were observed, except in three bile specimens where GDCA and/or TDCA were not detected (Table 4 serial Nos. 1, 4, and 11). Further, although there was a large variation between the relative and absolute quantities of bile acids, the ratio of median values of taurine- to glycine-conjugated bile acids was 1:4 for primary bile acids (CA and CDCA) whereas the ratio was 1:5.7 for the secondary bile acid (DCA). These results show the potential of NMR for determining the individual conjugated bile acids rapidly in a single step. Furthermore, it is even possible to analyze unconjugated bile acids, if present. This can be achieved from the presence or absence of carbonyl carbon signal(s) near 187 ppm (26). The chemical shift region of the carboxylic carbons for unconjugated bile acids, which normally does not contain any other bile component signals, serves as a marker. In the ^{13}C spectrum of typical bile shown in Figure 4, the absence of such signals (near 187 ppm) clearly indicates the absence of any unconjugated bile acids (under the detection limits of present experimental conditions). Although variations in the bile acids, as shown in Table 4, does not seem to show any direct correlation with the diagnosis, detailed and systematic studies are required to relate these variations to the underlying pathophysiology of the hepatobiliary diseases.

In summary, we present here ^{1}H and ^{13}C NMR chemical shift assignment of intact bile under natural conditions and identified six major conjugated bile acids. Accurate and rapid analysis of all the conjugated bile acids in a single step presented herein is significantly advantageous over other methods. Moreover, the method is simple and does not need any sample processing. This may have significant impact on the studies of bile acid synthesis, metabolism, and associated hepatobiliary and gastrointestinal diseases. Studies using the method presented herein are currently in progress on bile specimens from a large number of patients with gallbladder disease, to have better understating of the diseases of biliary origin.

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Accelerated Solvent Extraction for Quantitative Measurement of Fatty Acids in Plasma and Erythrocytes

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ABSTRACT: Consumption of fish rich in n-3 highly unsaturated FAs (i.e., EPA and DHA) has been suggested to decrease the risk of lifestyle-related diseases such as coronary heart disease, cancer, diabetes, and dementia. Blood levels of those FA are known appropriate biomarkers of both the corresponding dietary FA intakes and fish consumption. In place of traditional handwork methods for extracting FA, we performed an accelerated solvent extraction (ASE) for at least 13 selected FA in plasma and erythrocytes to measure them by GLC. The FA levels (concentrations and compositions) in 35–50 μL of plasma or erythrocytes were extracted by ASE and measured by GLC. Intra- and interassay coefficients of variation were ≤6.0% for both blood materials, except with a minor group of FA (≤1.0% of total FA). When ASE was compared with two traditional handwork methods, FA levels in plasma from 18 healthy subjects were all coincident with very high Pearson's correlation coefficients for the three sets of the same 18 samples ($r \geq 0.85$ to 0.95, $P < 0.0001$), except for 18:0 ($r = 0.59$, $P < 0.01$). Using ASE and GLC, we have developed a new method for determination the levels of FA in plasma and erythrocytes as biomarkers for dietary intake of fish, fat, and FA. This new method makes it feasible to measure small volumes of samples, automatically, quantitatively, routinely, easily, rapidly and cheaply, with acceptable precision and accuracy.

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A high-fat diet, especially one rich in animal fat or saturated FA, is suggested to increase the likelihood of obesity and the development of so-called lifestyle-related diseases, such as hyperlipidemia, coronary heart disease, cancers in several sites (colorectum, breast, and prostate), diabetes, dementia, and allergies (1–7). Furthermore, n-6 PUFA such as 20:4n-6 (arachidonic acid) are converted into leukotrienes, prostaglandins, and thromboxanes, and those eicosanoids are suggested to cause arthritis, asthma, cell proliferation, thrombosis, vasospasm, and inflammatory disorders (7–9). On the other hand, consumption of fish and n-3 highly unsaturated FA (HUFA = 20:5n-3 [EPA] + 22:5n-3 + 22:6n-3 [DHA]) exhibits inverse relationships with such diseases (10–19). In contrast to findings based on only dietary assessment, we have demonstrated that increased and decreased risks of colorectal cancer are linked to high compositions of palmitic acid and DHA, respectively, in erythrocyte membranes (20). For accurate assessment of impact on risk, therefore, we think it essential to measure FA in biomaterials (2,19,21–23).

Blood levels (concentrations [mg/dL or mmol/L] and compositions [wt% or mol%]) of EPA, DHA, and n-3 HUFA are known as appropriate biomarkers of dietary intake of FA from fish (24–31). Traditional handwork methods (THM) for measuring FA in biomaterials have gradually been improved (32–38), but require the following common multiple steps (Fig. 1): (1) pretreatment of biomaterial samples; (2) extraction of lipids (triglycerides, cholesterol esters, and phospholipids) and free FA (32–34); (3) conversion from lipids to FA, and then to methyl esters (trans-methylation) (35,36); (4) extraction of methyl esters; and (5) measurement by GLC (37,38). In the first step, lipids and free FA can be separated by silica gel TLC (33,34). The precision for measuring FA is determined primarily by the second and fourth steps, and a total of three extractions with each organic solvent at each of the steps is required to maximize recovery. It takes several hours to perform the extraction procedure by hand with considerable physical effort. Hitherto, two different automatic extraction methods for FA from small numbers of samples of plasma and foodstuffs such as meat have been reported

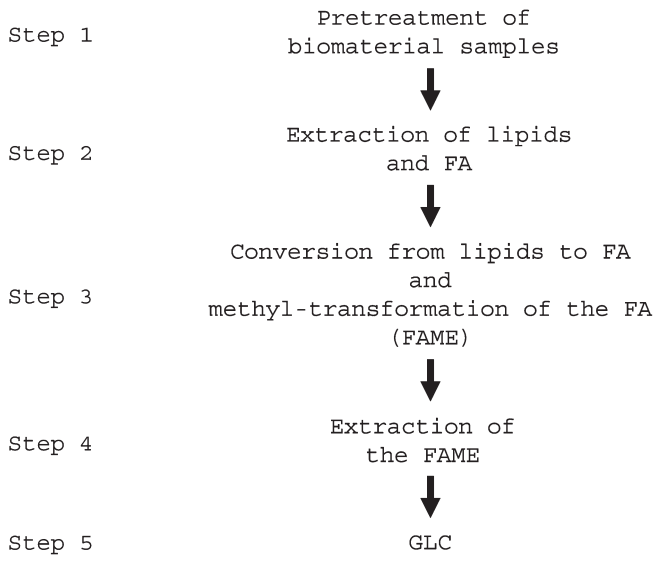

FIG. 1. Procedure for a representative traditional handwork method for measuring FA.

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Abbreviations: ASE, accelerated solvent extraction; HUFA, highly unsaturated FA; THM, traditional handwork method.

(39,40), but we do not have enough information about such methods for biomaterials such as plasma and erythrocytes for clinical use or epidemiological investigations.

To improve the methodology for measuring FA in biomaterials, especially at the second and the fourth steps (Fig. 1), we have established an automatic extraction method featuring accelerated solvent extraction (ASE). Compared with two THM, we here demonstrate high precision and accuracy for quantitative measurement of FA levels (concentrations and compositions) in small volumes of plasma and erythrocytes by using this method.

MATERIALS AND METHODS

Samples and standards were systematically protected from contamination with both natural existing FA and detergents and from auto-oxidation by direct light and oxygen in air during sample processing. Most manipulations and storage were performed in glass tubes or bottles and handling was with glass pipettes (Pasteur pipettes) for organic solvents, to avoid the influence of plasticizers. A 10.0 μL quantity of an internal standard was pipetted with a micro glass syringe (SGE, Victoria, Australia), and the variation in accuracy (CV, %) for a series of 10 samples was ≤5%.

Reagents. We used the following reagents: petroleum ether (containing 20–30% of *n*-hexane), decane (Wako, Osaka, Japan), chloroform, methanol (Kanto Kagaku, Tokyo, Japan), sodium carbonate, disodium hydrogenphosphate dodecahydrate, and sodium dihydrogenphosphate dehydrate (Sigma, Tokyo, Japan). As standard chemicals, the following FA were obtained from Sigma: 14:0 (myristic acid), 16:0 (palmitic acid), 16:1n-7 (palmitoleic acid), 18:0 (stearic acid), 18:1n-9 (oleic acid), 18:2n-6 (linoleic acid), 18:3n-6 (γ-linolenic acid), 18:3n-3 (α-linolenic acid), 20:3n-6 (dihomo-γ-linolenic acid), 20:4n-6, EPA, 22:5n-3 (docosapentaenoic acid), and DHA. 17:0 (*n*-Heptadecanoic acid), BHT, and hydrogen chloride methanol reagent 10 (10–20% hydrochloride) were used as an internal standard, an antioxidant reagent, and a methyltransformation reagent (Tokyo Kasei Kogyo, Tokyo, Japan), respectively. Stock amounts of the internal standard and other standard chemicals were weighed on an analytical balance (with 0.01 mg precision), dissolved in decane containing BHT, then stored in Teflon-lined, screw-capped test tubes at 4°C. Stock solutions of BHT and decane were also made and stored at 4°C.

Blood materials. The Ethics Committee of each relevant institute approved the following three studies. First, in the framework of the Dietary Intervention to Polypectomized Patients (DIPP) study (41) and the Japanese Dietitians' Epidemiologic (JADE) study (29,30,42,43), we collected and stocked pooled plasma as a control, as described elsewhere (44). Plasma from anonymous patients with nonspecific medical conditions in Nagoya City University Hospital was gathered in one glass beaker on ice, mixed, and then poured into 1.5- to 5-mL stock tubes and stored at –80°C. We here used the pooled plasma for establishing measurement conditions for FA levels (mmol/L and mol%) in plasma according to the ASE.

Second, in the framework of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC) (45), we routinely collect blood materials from patients in Aichi Cancer Center Hospital (20). Blood was collected using EDTA-2Na tubes and centrifuged at 2,000 *g* for 15 minutes at 4°C. Plasma and erythrocytes were prepared and stored at –80°C until analysis of individual FA by GLC. They were provided with an explanatory document and all gave their written informed consent for participation in the study. We here used erythrocytes from three noncancer controls for establishing the measurement conditions for FA composition (mol%) of erythrocytes according to ASE.

Third, in the framework of the Japanese Dietitians' Epidemiologic (JADE) study (29,30,42,43), we collected overnight fasting blood from 106 middle-aged Japanese dietitians (21 men and 85 women). Likewise, plasma and erythrocytes were simultaneously prepared in tubes including EDTA-2Na and stored at –80 °C until analysis of FA by GLC. We collected informed consent for participation in this study from all subjects. We here used plasma from nine male and nine female dietitians for comparison with two THM for measuring FA levels (mmol/L and mol%) in plasma as described herein. The 18 subjects were randomly selected and had no bias, and the samples were of sufficient volume to accomplish our objectives.

Instrumentation. For our ASE system, we used an Accelerated Solvent Extractor (ASE®) 200 (Nippon Dionex, Osaka, Japan), approved for use by the U.S. Environmental Protection Agency SW-846 Method 3545A for Pressurized Fluid Extraction, and accepted under the Contract Laboratory Program, Statement of Work OLMO 4.2. The system is computerized and comprises a cell oven, a cell tray for applying samples, a vial tray for recovering extracted solutions, a solvent sending pump, connected by a bound flexible line to solvent reserved bottles, and a nitrogen gas cylinder. To reduce solvent consumption, the line was changed from the standard 1.0 mm i.d. to 0.5 mm i.d. As optional equipment, an ASE® Solvent Controller allows for automatic mixing and delivery of different solvents. A small-size reciprocal air compressor, Toscon SLP5D-2SV (Toshiba, Tokyo, Japan), was included as part of the driving power to achieve safety for highly inflammable volatile solvents. The ASE 200 also features a series of cells (1 mL, 5 mL, and so on) that are stainless steel vessels for extracting target compounds from samples. A cylindrical cell body and two caps for the upper and bottom ends comprise one cell.

The system is usually employed with the following manipulations for each sample: (1) placement of a filter paper (Nippon Dionex, Osaka, Japan) in the bottom of a cell; (2) introduction of the sample into the cell; (3) movement of the cell to a cell oven; (4) introduction of the extracting solvent into the cell; (5) maintenance of an elevated temperature and pressure; (6) transfer of extracted solution to a glass vial and purging the residue from the cell with nitrogen gas when the extraction is completed. The filtered extracts are then taken

away from the extracted solution. This equipment automatically extracts, one by one, a maximum of 26 samples in minutes, by using conventional solvents at elevated temperatures and pressures, and reduces solvent consumption compared with general THM.

The equipment for the GLC system was as follows: a Shimadzu GC-2010 with a flame ionization detector (FID-2010), an auto-injector AOC-20i, and an auto-sampler AOC-20s and a hydrogen generator OPGU-2100S (Shimadzu, Kyoto, Japan). Shimadzu GC workstation software, GC solution version 2 (Shimadzu, Kyoto, Japan), provides multisystem PC control and advanced sample tracking. An air line bypasses from the line of the extraction system. A silica capillary column (30 m × 0.25 mm i.d.; DB-225 coating thickness 0.25 μm) (J&W Scientific, Folsom, CA) was employed (29,37).

GLC analysis. The GLC analysis has been detailed elsewhere (29,37), but was slightly modified according to the version of our GLC system. In brief, the oven temperature was programmed from 140°C to 180°C at a rate of 20°C/min and then from 180°C to 240°C at 3°C/min, with holding of the final temperature for 23.5 min. Total run time for one sample was 45.5 min. At about 42.5 min, the fraction of cholesterol esters and its derivatives were eluted. The nitrogen carrier flow rate was 10.2 mL/min (pressure, 200.0 kPa as a control mode) and for the nitrogen makeup gas was 28.0 mL/min. The column flow rate was 2.41 mL/min (line speed, 53.0 cm/s; purge flow rate, 3.0 mL/min). For the flame ionization detector, flow rates of hydrogen and air were 40.0 and 400.0 mL/min, respectively. The temperatures of the injection port and detector were 250°C and 260°C, respectively. The auto-injector was programmed to rinse a 10-μL syringe, once for each sample, pump three times to remove air bubbles, and then inject 3.0 μL of sample with a split-less method (sampling time, 1.0 min). The syringe was then rinsed five times with solvent. Integration and calculation of peak areas were performed with GC solution version 2 software, and the chromatographs and data were electronically stored.

Evaluation of FA levels in biomaterials. The identity of individual FA peaks was ascertained by comparison of the peak retention times with those of authentic standards. A single-point calibration was performed with an aliquot of an internal standard stock solution and the linearity of the method was checked by additional calibrators with a series of an internal standard concentration and appropriate dilutions of the stock solution. With integration of areas under the individual peaks adjusted for that of an internal standard, each FA was quantified as an absolute concentration (mmol/L) in plasma and compositions (molar weight percentage [mol%]) of total FA in plasma and erythrocytes. The following 13 FA were referenced to previous reports (29,30,43,46), which were investigated for associations between dietary intake of fish, fat, and FA, serum levels of triglycerides, total and HDL cholesterols, and the blood levels of FA: 14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:2n-6, 18:3n-6, 18:3n-3, 20:3n-6, 20:4n-6, EPA, 22:5n-3, and DHA.

Compared with THM-A as described herein, recovery rates (%) for the same 13 standard chemicals (a series of 10 samples) were calculated after step 2, step 4, and all procedures of the new method featuring ASE (Fig. 1). Intra-assay CV were based on the analysis of a series of 10 samples, derived from one sample/subject of pooled plasma or erythrocytes, and then all extracted and analyzed within a day. Interassay CV were based on replicate analyses of pooled plasma over a period of 10 d, and a total of 100 samples (10 samples/d × 10 d) were measured. Interassay reproducibility (CV), moreover, was checked according to two replicate measurements of the same pooled plasma per day for 20 d (total 40 samples = 2 samples/d × 20 d). With erythrocytes, samples from three subjects were used as each control, because a large volume of erythrocytes could not be collected from a single subject.

Comparison of methodologies. The new method featuring ASE was compared with two different THM for measuring FA levels (mmol/L and mol%) using 18 plasma samples from one part of the Japanese Dietitians' Epidemiologic (JADE) study (42) as described here previously. The three methods are distinctly different regarding extraction, trans-methylation, and GLC analyses of FA, respectively. Using the following THM-A (29), we have demonstrated relationships between dietary intakes of fish, fat, and FA and the corresponding FA levels in plasma in the study (29,30,43). The same samples were also measured by the following THM-B (47) at a clinical laboratory testing service (SRL Inc., Tokyo, Japan). We have no conflict of interest or collaborative agreement with the service company. The samples, therefore, were independently measured for us.

As THM-A (29), measurement was with the same reagents and procedures, except for the following: (1) the required sample volume was 100.0 μL; (2) the proportion of chloroform in the extracting solvent differed at the second step (Fig. 1); (3) each organic layer at the second and fourth steps was extracted from water-mixed phases by centrifugation. In brief, after 100.0 μL of plasma was transferred into a glass tube with a Teflon-lined screw-cap, 10.0 μL of an internal standard and 10 μL of BHT were added. Then 700 μL of 0.9 wt/vol% potassium chloride aqueous solution and 3 mL of Folch's solvent (chloroform/methanol, 2:1, vol/vol) (32) were added followed by vigorous mixing. After standing for 10 min at room temperature, 1 mL of water and 1 mL of chloroform were added with vigorous mixing, and then the lipids and free FA were extracted by centrifugation (2,000 *g*, 20°C, 5 min). The bottom chloroform layer was collected by using a glass pipette, and then the extraction was repeated a total of three times to maximize recovery with 2 mL of chloroform. The extracted solutions were pooled and evaporated to dryness under nitrogen gas, and then converted to FAME by 1 mL of hydrogen chloride methanol reagent 10 (10–20% hydrochloride), at 100°C for 60 min (29). At the fourth step, likewise, a total of three extractions with 2 mL of petroleum ether were performed. The GLC equipment was a Shimadzu GC-17A with a hydrogen gas cylinder and a Chromatopac Integrator C-R7A (Shimadzu, Kyoto, Japan). Measurement condition of GLC has been reported elsewhere (29,37). Re-

garding precision of the FA measurements in plasma, intra- and interassay CV were distributed from 1.8% to 4.8% and from 2.5% to 7.2%, respectively (29).

For THM-B (47), the measurement principles and the steps (Fig. 1) were basically the same as THM-A (29). The equipment was also the same as THM-A, except for a hydrogen generator, HGE-1A (Shimsadzu, Kyoto, Japan). However, the lipids and free FA were first extracted with Folch's solvent (32), and FA were then treated with 100% chloroform after acid hydrolysis by 0.5 M hydrochloride and acetonitrile/water (9:1, vol/vol). Then, 0.4 N potassium methoxide (Kanto Kagaku, Tokyo, Japan) and 14.0 wt% of boron trifluoride–methanol (GL Sciences, Tokyo, Japan) were used for transmethylation of FA at the third step (30,35). The FAME were extracted with Wako-gel C-200, *n*-hexane (Wako, Osaka, Japan), and water at the fourth step. The conditions of centrifugation were 2,000 *g* at 20 °C for 5 min. A fused silica capillary column (30 m × 0.25 mm i.d.; Omegawax 250 coating thickness 0.25 μm) (Supelco, Bellefonte, PA) was employed. For FA measurements in plasma, intra- and interassay CV were distributed from 2.1% to 8.1% and from 5.5% to 8.7%, respectively.

Statistics. Statistical analyses were performed with PC-SAS version 9.1 (SAS Institute Inc., Cary, NC). The data are given as mean ± SD. Pearson's correlation coefficients between the values (mmol/L and mol%) for plasma FA were calculated. All tests were two-sided and a P value of 0.05 or less was taken as significant.

RESULTS

Sample preparation for ASE. When plasma samples were directly introduced into a 1-mL cell for extraction with organic solvent on ASE 200, the extraction at the second step (Fig. 1) needed to be discontinued because very small particles of aggregated soluble proteins in plasma clogged the small exit of the cell bottom cap. As the first step, therefore, a membrane filter (OMMIPORE™, 0.2 μm, JG) (Millipore, Cork, Ireland) was firmly fixed between a cell body and the cell bottom cap to prevent clogging, and then two paper filters were laid on the membrane filter. This manipulation is not described in the manual for using ASE 200 and was our original idea. To prevent elution or contamination with FA, moreover, we washed the paper filters in the extracting solvent before use. The procedure for applying one plasma sample was as follows: (1) 10.0 μL of the internal standard (3.5 mmol/L) and 10 μL of BHT (0.45 mmol/L) were added into the cell; (2) 35.0 μL of plasma and 150 μL of extracting solvent (chloroform/methanol, 1:2, vol/vol) were vigorously mixed in a 1.5-mL micro tube, and then the solution was transferred into the cell; (3) 50 μL of extracting solvent was rinsed in the micro tube and then the residue was also added to the cell. Although we examined the range 25–50 μL of plasma sample for measurement, the optimal volume was 35.0 μL (data not shown). As described above, the extraction step from more than 50 μL of plasma may abort because of being clogged in the ASE 200 line due to aggregated soluble proteins. Two pieces of paper filter were used to absorb the internal standard and BHT, and the pretreated plasma sample, in that order.

To remove hemoglobin and prevent oxidative degradation of lipids and FA caused by iron of hemoglobin, erythrocytes were prepared to erythrocyte membranes (white ghosts) with 5 mM sodium phosphate buffer (pH 7.4) (48). The white ghosts from one sample were directly applied into a 1-mL cell put on one paper filter to extract. In this case, a membrane filter was not needed. As with plasma samples, 10.0 μL of the internal standard (1.0 mmol/L), 10 μL of BHT (0.45 mmol/L), and the white ghosts were added into the cell, in that order. The optimal volume of erythrocytes sample for measuring was 50.0 μL (data not shown). Less than 25 μL of erythrocytes could not be stably measured under our current conditions, while more than 100 μL required much increased rinsing time with sodium phosphate buffer to make white ghosts. It should be noted that FA contents in whole blood are not equivalent to those in erythrocyte membranes.

ASE for lipids, FA, and FAME extraction. After systematic changing in the conditions for ASE 200 at the second step (Fig. 1), the optimized conditions for extracting lipids and FA from blood materials were achieved as summarized in Table 1. There were the same for plasma and erythrocytes. Chloroform/methanol, 1:2 vol/vol, was used for the extraction. We extracted all 13 selected FA in blood materials at one time because the residual material was limited, as determined by repeated extraction tests. For one sample, the volume of the extracted solution

TABLE 1
Optimized Conditions for Extracting Lipids and FA at the Second Step and FAME at the Fourth Step with an Accelerated Solvent Extractor (ASE® 200)

	Lipids and FA (at the second step)	FAME (at the fourth step)
Extraction cell (mL)	1	5
Heating time (min)	0	0
Static time (min)	5	0
Temperature (°C)	75	50
Pressure (psi)	1,500	1,500
Extracting solvent	Chloroform/methanol (1:2, vol/vol)	Petroleum ether
Flash (%)	10% of extracting solvent	10% of extracting solvent
Purge (s)	30	30

and the extracting time were about 5.5–6 mL and 8.5 min, respectively. The time included rinsing of the line for ASE 200. The extracted solution was evaporated under a stream of nitrogen gas at 37°C.

All samples at step 3 (Fig. 1) were transferred into glass tubes with Teflon-lined screw-caps and then treated with 1 mL of hydrochloride-methanol reagent for FA conversion from lipids and subsequent methyl-transformation. The glass tubes were tightly capped and placed in a block heater (100°C), and then withdrawn and cooled after 60 minutes of heating. To prevent erosion of the stainless steel cells by the potent hydrochloric acid, 0.8–2 mL of 0.4 M sodium carbonate aqueous was added into the tubes and the solution was prepared to pH 7. The volume of aqueous sodium carbonate corresponded to 5–15% concentrations (depending on lot numbers of the products) of hydrochloride. In GLC analyses, alkaline hydrolysis of FAME caused by the addition of sodium carbonate aqueous was not observed (data not shown).

At the fourth step (Fig. 1), petroleum ether was used as an organic solvent for extracting FAME from the water-methanol layer on ASE 200, but the layer was not directly applied into a 5-mL cell because of leakage through the exit of the bottom cap. To prevent this, therefore, a membrane filter was firmly fixed between the cell body and the cell bottom cap. This manipulation is not described in the manual for using ASE 200 and was our original idea. Paper filters were not used here. First, the entire water-methanol layer was transferred into the cell. Second, the glass tube was rinsed with 500 μL of petroleum ether and then the residue was also added to the cell. After systematic changes, the optimized conditions for extracting methyl esters by ASE 200 are summarized in Table 1. In this step, we also extracted all 13 selected FAME at one time because residues were limited according to repeated extraction tests. For one sample, the volume of extracted solution and the extracting time (including rinsing time) were about 7.5–8 mL and 3.5 min, respectively.

GLC separation of individual FA. The extracted solution at the fourth step (Fig. 1) was evaporated under a stream of nitrogen gas at 37°C for complete removal of the solvent, and then the residue was redissolved in 400.0 μL of petroleum ether for application to GLC. Representative GLC profiles for individual FA in plasma and erythrocytes are shown in Figure 2. We could clearly demonstrate separation of individual FA.

Precision and accuracy. According to the analysis of a series of 10 samples, recovery rates of the internal standard were 86.4% after step 2, 97.6% after step 4, and 81.6% after all procedures with the new method featuring accelerated solvent extraction. For the 13 selected standard chemicals, however, the ranges of recovery rates (%) adjusted for an internal standard were distributed from 95.6% (for 18:3n-6) to 107.9% (18:0), 95.1% (18:0) to 102.1% (18:3n-6), and 94.5% (18:3n-6) to 109.1% (18:2n-6), in that order. Table 2 shows FA levels (mol/L and/or mol%) in blood materials and the intra- and interassay CV according to the new method. For

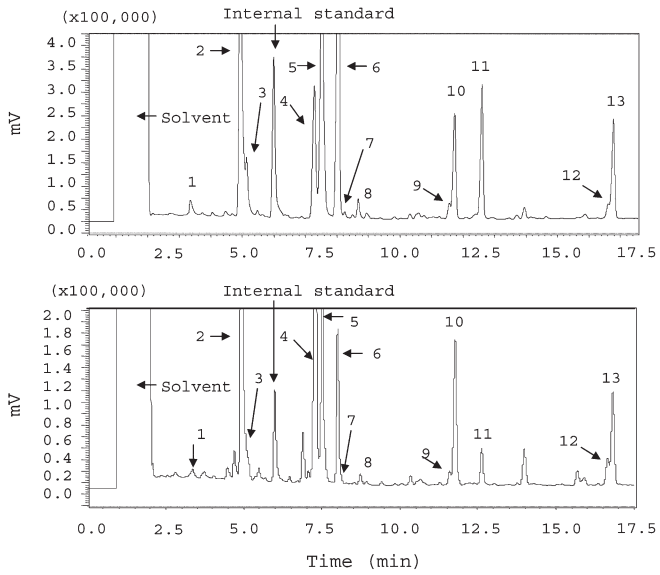

FIG. 2. Representative chromatograms after gas-liquid chromatography for the 13 selected FA in plasma (top) and erythrocytes (bottom). Peaks: internal standard (n-neptadecanoic acid, 17:0); 1, myristic acid (14:0); 2, palmitic acid (16:0); 3, palmitoleic acid (16:1n-7); 4, stearic acid (18:0); 5, oleic acid (18:1n-9); 6, linoleic acid (18:2n-6); 7, γ-linolenic acid (18:3n-6); 8, α-linolenic acid (18:3n-3); 9, dihomo-γ-linolenic acid (20:3n-6); 10, arachidonic acid (20:4n-6); 11, eicosapentaenoic acid (20:5n-3); 12, docosapentaenoic acid (22:5n-3); 13, docosahexaenoic acid (22:6n-3).

plasma, the intra-assay CV were ≤6.0%, except for the concentration and the composition of 14:0 (9.7% and 8.0%, respectively). The interassay CV for mean concentrations and compositions of the total 100 samples (10 samples × 10 d) were <6.0% for all 13 selected FA, and the distribution of the CV over a period of 10 d was <6.0%, except with the concentrations of 14:0, 16:1n-7, 18:3n-6, and 18:3n-3 (8.4, 6.8, 7.0, and 6.8%, respectively) and the composition of 14:0 (6.9%). For the compositions in erythrocytes, the intra-assay CV were also <4.0%, except a minor group of 14:0, 18:3n-6, and 18:3n-3 (≤0.5% of total FA for each). The interassay CV (total 100 samples) were also <4.0%, except for a minor group of 14:0, 18:3n-6, and 18:3n-3. We could not demonstrate CV for mean compositions because we could not collect sufficiently large volumes of erythrocytes from a single subject. Moreover, interassay reproducibility over a period of 20 d (the CV for a total 40 samples [2 samples × 20 d]) was almost the same distribution (data not shown).

Although we lack a gold standard method, the mean levels (mmol/L and mol%) and the SD for each FA were coincident and differences in values among the three different methods were not major (Table 3). For example, plasma concentrations of EPA, which are closely associated with dietary intake, were 0.20, 0.27, and 0.23 for the means and 0.12, 0.16, and 0.14 for the SD, in that order. The compositions were 1.7, 2.0, and 1.9, and 1.0, 1.1, and 1.0, respectively. We demonstrated, furthermore, that most of the Pearson's correlation coefficients between them were ≥0.95 ($P < 0.0001$) and all of them were

TABLE 2
Intra- and Interassay CV for FA Measurements in Plasma and Erythrocytes According to a New Method Featuring Accelerated Solvent Extraction

	Intra-assayCVs (10 samples within a day)		Inter-assay CVs: Total 100 samples (10 samples/d × 10 d)		Inter-assay CVs: Distribution of the CVs (10 d for 100 samples)
	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD
Concentration (mmol/L) in plasma					
14:0	0.10 ± 0.010	9.7	0.10 ± 0.005	4.6	8.4 ± 1.5
16:0	2.67 ± 0.102	3.8	2.63 ± 0.068	2.6	4.4 ± 0.9
16:1n-7	0.35 ± 0.016	4.7	0.35 ± 0.015	4.4	6.8 ± 1.4
18:0	0.77 ± 0.039	5.0	0.77 ± 0.025	3.3	4.7 ± 1.3
18:1n-9	2.50 ± 0.111	4.4	2.46 ± 0.073	3.0	5.6 ± 1.0
18:2n-6	2.84 ± 0.120	4.2	2.79 ± 0.105	3.8	5.5 ± 1.0
18:3n-6	0.03 ± 0.002	6.0	0.03 ± 0.001	5.1	7.0 ± 1.6
18:3n-3	0.08 ± 0.004	4.7	0.08 ± 0.003	4.6	6.8 ± 1.6
20:3n-6	0.09 ± 0.002	2.4	0.09 ± 0.003	3.7	4.4 ± 1.4
20:4n-6	0.51 ± 0.018	3.5	0.51 ± 0.021	4.1	4.4 ± 1.0
20:5n-3	0.16 ± 0.006	4.0	0.16 ± 0.008	5.2	5.0 ± 1.1
22:5n-3	0.04 ± 0.002	4.5	0.04 ± 0.002	5.8	5.0 ± 1.2
22:6n-3	0.32 ± 0.013	4.1	0.33 ± 0.018	5.6	4.4 ± 1.0
Composition (mol%) in plasma					
14:0	1.0 ± 0.08	8.0	1.0 ± 0.05	4.8	6.9 ± 2.0
16:0	25.6 ± 0.21	0.8	25.5 ± 0.19	0.8	0.9 ± 0.2
16:1n-7	3.3 ± 0.09	2.6	3.4 ± 0.08	2.3	2.9 ± 0.8
18:0	7.4 ± 0.35	4.7	7.5 ± 0.12	1.6	3.6 ± 1.3
18:1n-9	23.9 ± 0.31	1.3	23.8 ± 0.17	0.7	1.1 ± 0.2
18:2n-6	27.2 ± 0.26	0.9	27.0 ± 0.26	1.0	0.9 ± 0.1
18:3n-6	0.3 ± 0.01	3.3	0.3 ± 0.01	3.3	3.6 ± 0.9
18:3n-3	0.7 ± 0.03	4.3	0.7 ± 0.03	3.4	5.6 ± 2.4
20:3n-6	0.8 ± 0.02	2.4	0.8 ± 0.02	2.5	2.2 ± 0.7
20:4n-6	4.8 ± 0.06	1.3	4.9 ± 0.09	1.8	1.4 ± 0.2
20:5n-3	1.5 ± 0.02	1.1	1.6 ± 0.04	2.8	1.1 ± 0.3
22:5n-3	0.4 ± 0.01	2.5	0.4 ± 0.02	3.9	3.1 ± 0.7
22:6n-3	3.1 ± 0.06	2.0	3.2 ± 0.11	3.5	2.2 ± 0.3
Composition (mol%) in erythrocytes					
14:0	0.5 ± 0.05	10.4			13.1 ± 7.4
16:0	27.8 ± 0.23	0.8			1.3 ± 0.5
16:1n-7	1.6 ± 0.03	2.0			2.7 ± 1.3
18:0	18.5 ± 0.56	3.0			2.3 ± 0.6
18:1n-9	16.1 ± 0.61	3.8			3.4 ± 0.7
18:2n-6	10.9 ± 0.41	3.8			3.3 ± 1.5
18:3n-6	0.02 ± 0.002	7.7			19.2 ± 8.2
18:3n-3	0.2 ± 0.03	18.1			15.6 ± 5.8
20:3n-6	0.9 ± 0.01	1.4			1.5 ± 0.4
20:4n-6	11.4 ± 0.43	3.8			2.0 ± 0.8
20:5n-3	2.2 ± 0.04	1.8			2.9 ± 1.8
22:5n-3	2.4 ± 0.04	1.6			2.4 ± 0.9
22:6n-3	7.4 ± 0.10	1.3			2.2 ± 0.8

at least ≥0.85 ($P < 0.0001$), except for the composition of 18:0 (Table 4). The reason for the relatively low coefficients in this case was unclear, but the level with the new method featuring ASE significantly correlated with that with THM-A ($r = 0.72$, $P < 0.001$).

DISCUSSION

Some PUFA are particularly useful in assessing the corresponding intakes in the short term in plasma and serum, in the medium term for phospholipids in erythrocytes and platelets, and in the long term for adipose tissue (24,25), because they are not biosynthesized in humans. Especially the levels of EPA, DHA, and n-3 HUFA are appropriate biomarkers of the relative dietary intakes (26–31), but they have not routinely been used due to laborious handwork for measurement, which also means expense in the commercial setting. Using ASE, however, here we could develop a new method featuring ASE for measuring FA in plasma and erythrocytes that demonstrated acceptable precision and accuracy, with many practical advantages.

TABLE 3
Comparison of FA Levels in Plasma from 18 Healthy Subjects Measured by a New Method Featuring Accelerated Solvent Extraction, and Traditional Handwork Methods (THM)-A and -B

	New method Mean ± SD	THM-A1 Mean ± SD	THM-B[a] Mean ± SD
Concentration (mmol/L) in plasma[b]			
14:0	0.11 ± 0.05	0.13 ± 0.08	0.16 ± 0.10
16:0	2.69 ± 0.70	3.12 ± 0.95	3.00 ± 0.96
16:1n-7	0.86 ± 0.11	0.83 ± 0.10	0.73 ± 0.10
18:0	0.91 ± 0.21	0.97 ± 0.28	0.90 ± 0.27
18:1n-9	2.37 ± 0.74	2.70 ± 0.82	2.18 ± 0.79
18:2n-6	3.18 ± 0.76	3.75 ± 0.88	3.54 ± 1.05
18:3n-6	0.03 ± 0.02	0.04 ± 0.02	0.04 ± 0.03
18:3n-3	0.11 ± 0.05	0.13 ± 0.06	0.11 ± 0.05
20:3n-6	0.08 ± 0.03	0.12 ± 0.04	0.10 ± 0.03
20:4n-6	0.56 ± 0.12	0.71 ± 0.18	0.59 ± 0.15
20:5n-3	0.20 ± 0.12	0.27 ± 0.16	0.23 ± 0.14
22:5n-3	0.05 ± 0.02	0.08 ± 0.02	0.07 ± 0.02
22:6n-3	0.37 ± 0.12	0.54 ± 0.17	0.46 ± 0.16
Composition (mol%) in plasma			
14:0	0.9 ± 0.3	0.9 ± 0.4	1.2 ± 0.5
16:0	23.3 ± 2.1	23.1 ± 2.4	24.5 ± 2.6
16:1n-7	7.7 ± 1.6	6.5 ± 1.5	6.4 ± 1.6
18:0	7.9 ± 0.8	7.2 ± 0.7	7.5 ± 0.9
18:1n-9	20.3 ± 2.7	19.9 ± 2.5	17.6 ± 2.5
18:2n-6	27.8 ± 3.6	28.3 ± 3.9	29.5 ± 4.2
18:3n-6	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.2
18:3n-3	0.9 ± 0.2	1.0 ± 0.3	0.9 ± 0.3
20:3n-6	0.7 ± 0.1	0.9 ± 0.2	0.8 ± 0.2
20:4n-6	4.9 ± 0.7	5.3 ± 0.9	5.0 ± 0.9
20:5n-3	1.7 ± 1.0	2.0 ± 1.1	1.9 ± 1.0
22:5n-3	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.2
22:6n-3	3.2 ± 0.9	4.0 ± 1.0	3.8 ± 1.0

[a]THM-A and THM-B are described in text.
[b]Plasma samples from 18 healthy subjects (9 men and 9 women) were measured for each of three different measurement methods. The sample selection is described in text.

TABLE 4
Pearson's Correlation Coefficients Between Levels of FA in Plasma from 18 Healthy Subjects Determined by a New Method Featuring Accelerated Solvent Extraction, and Traditional Handwork Methods (THM)-A and -B

	Between new method and THM-A[a]		Between new method and THM-B[a]		Between THM-A and THM-B	
	r	P	r	P	r	P
Concentration (mmol/L) in plasma[b]						
14:0	0.97	<0.0001	0.99	<0.0001	0.97	<0.0001
16:0	0.96	<0.0001	0.93	<0.0001	0.96	<0.0001
16:1n-7	0.99	<0.0001	0.91	<0.0001	0.93	<0.0001
18:0	0.94	<0.0001	0.93	<0.0001	0.97	<0.0001
18:1n-9	0.96	<0.0001	0.88	<0.0001	0.92	<0.0001
18:2n-6	0.94	<0.0001	0.89	<0.0001	0.91	<0.0001
18:3n-6	0.97	<0.0001	0.97	<0.0001	0.98	<0.0001
18:3n-3	0.97	<0.0001	0.95	<0.0001	0.99	<0.0001
20:3n-6	0.96	<0.0001	0.98	<0.0001	0.97	<0.0001
20:4n-6	0.96	<0.0001	0.95	<0.0001	0.96	<0.0001
20:5n-3	0.99	<0.0001	0.99	<0.0001	0.99	<0.0001
22:5n-3	0.96	<0.0001	0.97	<0.0001	0.98	<0.0001
22:6n-3	0.97	<0.0001	0.98	<0.0001	0.98	<0.0001
Composition (mol%) in plasma						
14:0	0.97	<0.0001	0.99	<0.0001	0.97	<0.0001
16:0	0.97	<0.0001	0.95	<0.0001	0.97	<0.0001
16:1n-7	0.97	<0.0001	0.95	<0.0001	0.97	<0.0001
18:0	0.72	<0.001	0.59	<0.01	0.78	<0.001
18:1n-9	0.98	<0.0001	0.91	<0.0001	0.93	<0.0001
18:2n-6	0.98	<0.0001	0.97	<0.0001	0.96	<0.0001
18:3n-6	0.96	<0.0001	0.96	<0.0001	0.98	<0.0001
18:3n-3	0.97	<0.0001	0.96	<0.0001	0.99	<0.0001
20:3n-6	0.93	<0.0001	0.91	<0.0001	0.85	<0.0001
20:4n-6	0.98	<0.0001	0.89	<0.0001	0.91	<0.0001
20:5n-3	1.00	<0.0001	0.99	<0.0001	1.00	<0.0001
22:5n-3	0.96	<0.0001	0.94	<0.0001	0.97	<0.0001
22:6n-3	0.98	<0.0001	0.96	<0.0001	0.96	<0.0001

For footnotes, see Table 3.

ASE 200 is capable of concentrating small amounts of object chemicals and compounds rapidly, such as herbicides, pesticides, polychlorinated biphenyls (PCB), and dioxins, diluted in environmental media (soil and foods), according to the ASE kinetics made possible by increased temperature while keeping the solvent below its boiling point under pressure. We here demonstrated quantitative extraction of FA from ≤50 μL of blood materials, with acceptable values for accuracy and precision, intra- and interassay CV ≤6.0%, except for a minor group of FA, where most were <10.0%. According to our new method, the mean values of 20:3n-6, 22:5n-3, and DHA in plasma samples were about 20% lower than those with THM-A and B. As well as other 10 FA, however, the recovery rates for the three corresponding standard chemicals were adequately high and were 102.0, 97.9, and 97.1%, in that order. Therefore, we did not clarify the reason. Recovery experiments with repeated extraction also indicated that a single step does not result in large residues. Therefore, we just select the extraction program at the second and the fourth steps (Fig. 1) and push the start button after applying samples to cells of the ASE 200 for extraction of FA and FAME.

Even with our new method there are some limitations to routine measurement of FA in biomaterials. The series of necessary steps cannot be completely automated, and blood materials cannot be applied directly to the ASE system. It takes about 7 h for an extraction as a "day run" and 10 h for analysis as a "night run" to measure 12 samples of biomaterials. However, the method is applicable to homogenates of tissues and silica gel lipid fractions (triglycerides, cholesterol esters, phospholipids, and free FA) separated by TLC. Advantages, similarities, and disadvantages between the new method featuring ASE, THM-A, and B are summarized in Table 5. Compared with THM-A and B, our new method is performed easily without major physical effort, according to the automatic extraction of FA with ASE 200. Although total run time for 12 samples does not differ among the three different methods, our new method has an excellent time performance. However, the speed for extracting lipids or methyl esters, one by one, is a limitation with ASE 200. In the future, therefore, we still hope to make further improvements. The cost for a series of ASE 200 instruments is large, but there are no differences in the running costs for disposables and reagents

TABLE 5
Summary of the Differences for Measuring FA in Plasma and Erythrocytes According to a New Method Featuring Accelerated Solvent Extraction, THM-A, and THM-B

	New method	THM-A[a]	THM-B[a,d]
Instruments			
GLC for analyzing FA	Use	Use	Use
ASE 200 for extracting FA	Use	No use	No use
Centrifugal machines for			
pretreatment of plasma and erythrocytes	Use	Use	Use
1st and 2nd extraction	No use	Use	Use
Sample volume (mL)			
Plasma	35	100	100
Erythrocytes	50	50	(50)
Centrifugation (times)[b]			
Pretreatment of plasma and erythrocytes			
Plasma	12	0	0
Erythrocytes	36	36	36
Extraction steps			
1st extraction	0	36	36
2nd extraction	0	36	36
Manipulation[c]			
Pretreatment of plasma	–	+	+
Pretreatment of erythrocytes	±	±	±
1st extraction	++	–	–
Trans-methylation	+	+	–
(Number of processes)	(1 process)	(1 process)	(2 processes)
2nd extraction	++	–	–
GLC analysis	±	±	±
Run time (min)[b,c,d,e]			
Pretreatment of plasma	30	0	0
Pretreatment of erythrocytes	90	90	90
1st extraction	100	120	(120)
Methyl-transformation	60	60	(90)
2nd extraction	60	90	(90)
GLC analysis	±	±	±
Summary[c]			
Manipulation	++	–	–
Automatic extraction of FA	++	–	–
Non-considerable physical effort	++	–	–
Total run timee	±	±	±
Time performance	++	–	–
Treated sample number per day	±	±	±
Running costs	±	±	±
Cost for instruments	–	±	±

[a]THM-A and THM-B are described in detail in text.
[b]The figures for measuring FA of 12 samples are shown.
[c]Advantages (2 categories), similarities, and disadvantages between a new method, THM-A, and THM-B are here defined as ++ and +, ±, and –, in that order.
[d]When FA in plasma and erythrocytes were measured by THM-B in our laboratory, but not a clinical laboratory company (see the text), the estimated time is shown in parentheses.
[e]Run time is not included for some procedures, such as administration of an internal standard, an antioxidant chemical, and blood samples, and evaporation of the extracted solvents after the two extraction steps.

among the three methods. The commercial laboratory fee is very expensive.

In conclusion, using ASE and GLC, we have developed a new method for determining both the concentrations and the compositions of FA in plasma and erythrocytes as biomarkers for dietary intake of fish, fat, and FA. The advantages of our new method featuring ASE are that it is feasible to measure small volumes of multiple samples, automatically, quantitatively, routinely, easily, rapidly, and cheaply, with acceptable precision and accuracy. As a part of the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC) research, it will be a useful aid for the large numbers of blood samples available for Aichi Fatty Acid (AiFat) Research (20) necessitating routine measurement to clarify associations with cancers in different sites.

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HPLC Method for Quantification and Characterization of Cholesterol and Its Oxidation Products in Eggs

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ABSTRACT: A new method was developed for the simultaneous determination of cholesterol and its oxidation products in eggs, using HPLC with UV and refractive index (RI) detectors, and HPLC interfaced with atmospheric pressure chemical ionization coupled to MS (HPLC-APCI-MS). The best conditions for direct saponification of the sample and extraction of the nonsaponifiable material were defined using complete factorial designs with central points. The method showed accuracy and precision with a detection limit between 0.002 and 0.079 μg/g. The oxides cholest-5-ene-3β,20α-diol and cholest-5-ene-3β,25-diol identified by HPLC-UV-RI were not confirmed by HPLC-APCI-MS.

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Cholesterol takes part in essential functions of the human organism, but it is unstable, especially in the presence of light, heat, oxygen, moisture, radiation, low pH, or metals, producing oxides that may have adverse effects on human health. Cholesterol oxides are considered to be atherogenic and are related to inflammatory processes, rheumatoid arthritis, cytotoxicity, carcinogenesis, alterations in cell membrane properties (1), diabetes, and degenerative diseases such as Parkinson's and Alzheimer's (2,3). In food, cholesterol oxides are formed by a non-enzymatic oxidation process or autoxidation, which occurs by way of complex chain reactions based on the formation of free radicals with triplet and singlet oxygen (4). The consumption of these oxides and their possibly damaging effects have led to the need to develop methodology to identify and quantify these compounds in foods.

The cholesterol oxide contents found in the literature vary widely, due mainly to the analytical methodology used. HPLC and GC are frequently used to analyze the oxides. However, for analysis by GC, the samples need to be derivatized, which increases the time of analysis; heat destruction of cholesterol and hydroperoxides can also occur, with the formation of artifacts (5). Thus HPLC has been used more frequently, as it is carried out in a shorter time, and at relatively low temperatures. In particular, HPLC interfaced with atmospheric pressure chemical ionization coupled to MS (HPLC-APCI-MS) is capable of detecting samples that exist in trace amounts (6–8).

The simultaneous determination of cholesterol and its oxides is complex, especially as the oxides are present in food in minute amounts and show very similar structures (9). Some cholesterol oxides of biological importance, such as 5,6α-epoxy-5α-cholestan-3β-ol (5,6α-epoxy), 5,6β-epoxy-5β-cholestan-3β-ol (5,6β-epoxy), and 5α-cholestane-3β,5,6β-triol (triol), do not possess adequate characteristics for UV absorption (10,11). Although the refractive index (RI) detector is less sensitive than the UV and GC, it is useful for the detection of these oxides. Due to the instability of cholesterol, a mild analytical methodology is required to avoid the formation of artifacts, as the presence of air, light, solvents containing peroxides, or heat treatment can promote the formation of such compounds (12).

The majority of the methods used to analyze cholesterol oxides in foods involve an initial extraction of the fat followed by saponification (13,14). Using the values for standard deviation, the coefficient of variation, the formation of artifacts, and linearity, Dionisi *et al.* (15) showed that direct saponification of the samples was more efficient than the methods in which the fat was extracted first.

In the present study, a new method was developed for the simultaneous determination of cholesterol and its oxides using HPLC with UV and RI detectors and confirmed by HPLC-APCI-MS. The pre-chromatographic steps were evaluated by response surface methodology. In addition, after validating the method, it was applied to eggs.

MATERIALS AND METHODS

Solvents and chemicals. The HPLC-grade solvents used came from Mscience (Darmstadt, Germany), and the analytical-grade solvents were obtained from Merck (Darmstadt, Germany). The HPLC-grade solvents were vacuum filtered through 0.22-μm Millipore membranes (Bedford, MA) before use. The cholesterol, cholest-5-ene-3β,19-diol (19-OH), cholest-5-ene-3β,20α-diol (20α-OH), [22*R*]-cholest-5-ene-3β,22-diol (22(*R*)-OH), [22*S*]-cholest-5-ene-3β,22-diol

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Abbreviations: RSD, relative standard deviation; DL, detection limits; QL, quantification limits; HPLC-APCI-MS, HPLC interfaced with atmospheric pressure chemical ionization coupled to MS; RI, refractive index; 19-OH, cholest-5-ene-3β,19-diol; 20α-OH, cholest-5-ene-3β,20α-diol; 22(*R*)-OH, [22*R*]-cholest-5-ene-3β,22-diol; 24(*S*)-OH, [24*S*]-cholest-5-ene-3β,24-diol; 22(*S*)-OH, [22*S*]-cholest-5-ene-3β,22-diol; 25-OH, cholest-5-ene-3β,25-diol; 7-keto, 3β-hydroxycholest-5-ene-7-one; 7β-OH, cholest-5-ene-3β,7β-diol; 7α-OH, cholest-5-ene-3β,7α-diol; 5,6α-epoxy, 5,6α-epoxy-5α-cholestan-3β-ol; 5,6β-epoxy, 5,6β-epoxy-5β-cholestan-3β-ol; triol, 5α-cholestane-3β,5,6β-triol.

(22(*S*)-OH), cholest-5-ene-3β,25-diol (25-OH), 3β-hydroxy-cholest-5-ene-7-one (7-keto), cholest-5-ene-3β,7β-diol (7β-OH), 5,6α-epoxy, 5,6β-epoxy, and triol standards were purchased from Sigma (St. Louis, MO). [24*S*]-cholest-5-ene-3β,24-diol (24(*S*)-OH) and cholest-5-ene-3β,7α-diol (7α-OH) were obtained from Steraloids (Newport, RI) and the standard reference material (SRM 1845, whole egg powder) from NIST (Gaithersburg, MD).

Samples. Commercial spray-dried whole egg powder, stored in the dark at a temperature of 25 ± 2°C, was used to optimize the methodology for the simultaneous determination of cholesterol and its oxides. After optimization and validation, the method was applied to fresh eggs, eggs enriched with n-3 PUFA, and recently prepared commercial spray-dried whole egg powder. The fresh eggs and eggs enriched with n-3 PUFA were purchased directly from a local egg-processing plant and the commercial spray-dried whole egg powder from the factory (Mizumotu Alimentos Ltda, Guapirama, Paraná, Brazil).

Determination of the moisture content. Moisture content was determined using the AOAC (16) methodology.

Optimization of the saponification and extraction procedure. The simultaneous determination of cholesterol and its oxides was based on the direct saponification of samples (15), using experimental complete factorial designs with central points to verify the best conditions for saponification (concentration of KOH in ethyl alcohol and saponification time) and extraction of the nonsaponifiable matter (type of solvent and number of extractions). Subsequently a complete factorial design was developed with the significant factors (concentration of KOH in ethyl alcohol and number of extractions), to optimize the methodology.

HPLC equipment and conditions. The HPLC system used was a Shimadzu (Kyoto, Japan) composed of a ternary pump (LAD10), a vacuum solvent delivery degasser, a thermostatted column compartment with cooling, and UV (SPD-10AV$_{vp}$) and RI (RID-10A) detectors. The analytical column used was a Nova Pack CN HP, 300 mm × 3.9 mm × 4 µm (Waters, Milford, MA) column, and the oven temperature was set at 32°C. A mobile phase of *n*-hexane:isopropyl alcohol (96:4, vol/vol) with an isocratic flow rate of 1 mL/min and a 20-min analysis time, was used to separate cholesterol from the following oxides: 19-OH, 20α-OH, 22(*R*)-OH, 24(*S*)-OH, 22(*S*)-OH, 25-OH, 7-keto, 7β-OH, 7α-OH, 5,6α-epoxy, and 5,6β-epoxy. To separate triol, *n*-hexane:isopropyl alcohol (90:10, vol/vol) was used with a flow rate of 1 mL/min and analysis time of 15 min. Triol, 5,6α-epoxy, and 5,6β-epoxy were determined by the RI detector, whereas the UV detector was used to determine cholesterol and the other oxides, with the absorbance measured at 210 nm. Identification of the cholesterol and cholesterol oxide peaks was done by comparison of the retention times of the sample peaks with those of the standards and by co-chromatography. Quantification was done by external standardization, the standard curves being constructed with 6 points using solutions of the standards, with concentrations varying from 1 to 100 µg/mL for the oxides, and from 2 to 2.5 mg/mL for cholesterol.

Mass spectrometry. HPLC-APCI-MS was used to confirm the identity of cholesterol and its oxides in the egg samples. HPLC was carried out using a Waters Alliance 2695 pump (Milford, MA) and the same chromatographic conditions as described in the previous section. The mass spectrometer used was a Qtrap (Applied Biosystems, Concord, Ontario, Canada) with a QqQ (linear ion trap) configuration. Injections of cholesterol and cholesterol oxide standards were used to optimize the conditions. The optimum conditions were: positive mode APCI; scan range *m/z* 250–500; 375°C; nitrogen as carrier gas (70 L/min), sheath gas (60 L/min), and curtain gas (30 L/min); nebulizer current set at 3,000 V; declustering potential at 30 V; and entrance potential at 9 V. Chromatograms were obtained in the selective ion monitoring (SIM) mode for the ions *m/z* 367, 369, 385, 401, and 403. To verify the accuracy of results found by HPLC-UV-RI, a comparison with the results obtained by HPLC-APCI-MS was carried out. For this purpose, 10 commercial spray-dried whole egg powder samples were prepared and injected for both HPLC-UV-RI and HPLC-APCI-MS.

Method validation. The analytical method was validated for recovery and repeatability; detection limits (DL) and quantification limits (QL) were carried out according to Long and Winefordner (17). Standard reference material (SRM 1845, NIST) was also used to validate cholesterol, as no certified reference material exists for the cholesterol oxides.

Statistical analysis. The experimental designs were analyzed by response surface methodology using the software Statistic 5.5 (Statsoft 2000, Santa Clara, CA). ANOVA was used for the HPLC results, also using the Statistic 5.5 software, and the means were compared by Tukey's test, significance being based on a 0.05 probability level.

RESULTS AND DISCUSSION

Optimization of the conditions for saponification and extraction. The conditions for saponification and extraction of the nonsaponifiable matter were optimized using a complete 2^2 factorial design with central points; the variables and levels studied were concentration of the KOH in ethanol (15, 20, and 25%) and number of extractions (2, 3, and 4).

An analysis of the results of this design showed that all the factors evaluated were significant ($P < 0.05$), with significant interaction ($P < 0.05$) between the concentration of KOH and the number of extractions for the oxides 7α-OH and 5,6β-epoxy.

A concentration of 15% KOH and four extractions showed a positive effect on the response for 5,6β-epoxy, but the interaction between the factors was shown to be negative. For 7α-OH, concentration of KOH of 15% also influenced positively the response for oxide, but the greatest number of extractions showed the opposite effect. The interaction between the factors showed a positive effect for 7α-OH. There was a significant effect ($P < 0.05$) for the number of extractions with the cholesterol results, that is, a positive response was obtained with the greatest number of extractions. However, the KOH

concentration (%) did not influence the cholesterol results and there was no interaction between the factors evaluated.

For 5,6β-epoxy, the best results were obtained by reducing the KOH concentration and increasing the number of extractions. The sensitivity of epoxides to alkaline pH has been already reported (18). In the present work loss of 7-keto was not observed, although alkaline hydrolysis, even when carried out at room temperature and in the dark, can result in severe losses (19,20). A reduction in the number of extractions would negatively affect 5,6β-epoxy and cholesterol.

Thus a complete 2^2 factorial design with central points using standard reference material for cholesterol was developed with the significant factors to optimize the methodology and evaluating the greatest possible number of extractions. Optimum saponification conditions were obtained with 15% KOH and five extractions of the nonsaponifiable matter, corresponding to the central points of the design. Only the results for the central points were similar to the value specified on the certificate (19 ± 0.2 mg cholesterol/g whole egg powder).

Thus the optimum methodology was established as follows: 1.0 g of sample plus 10 mL 15% KOH in absolute ethanol was shaken at 118 rpm on a shaker in the dark for 18 h. The solution was transferred to a 100-mL screw-topped test tube, 40 mL distilled water and 10 mL ethyl ether were added, and the solution was mixed on a vortex mixer for 1 min. After phase separation, the upper layer was transferred to a 125-mL separating funnel, and the extraction with ethyl ether was repeated four more times. A volume of 15 mL KOH (0.5 mol/L) was added to the combined ethereal phases in a separating funnel, and the ethereal solutions washed with 15-mL aliquots of aqueous sodium sulfate (0.47 mol/L). The upper phase was filtered through common filter paper containing anhydrous sodium sulfate and transferred to a 70-mL screw-topped tube, and the ether was evaporated off with a stream of N_2. The dry extract was dissolved in 1 mL of the mobile phase used for HPLC, filtered through 0.45-μm Millipore membranes, and immediately injected into the HPLC.

Simultaneous determination of cholesterol and cholesterol oxides by HPLC-UV-RI. Under the chromatographic conditions used by Baggio *et al.* (21), it was possible to separate cholesterol from the following oxides: 19-OH, 20α-OH, 22(*R*)-OH, 24(*S*)-OH, 22(*S*)-OH, 25-OH, 7-keto, 7α-OH, 7β-OH, 5,6α-epoxy, and 5,6β-epoxy. A more polar mobile phase, *n*-hexane/isopropyl alcohol (90:10, vol/vol), had to be used to separate triol. The oxides 5,6α-epoxy, 5,6β-epoxy, and triol were determined by RI because they contain no π electrons in their structures and therefore do not absorb in the UV region. Both the UV and RI detectors can be used to determine cholesterol and the other oxidation products, but UV was used because it is more sensitive and selective. Figures 1 and 2 show the chromatograms of the standards and of the powdered egg samples, using the UV and RI detectors, respectively. Figures 1B and 2B show that only 5 of the 12 oxides studied were found in the samples of commercial spray-dried whole egg powder. The retention times of the peaks obtained by HPLC-UV of the samples coincided with the retention times of 20α-OH and 25-OH, and were spiked by the respective standards solutions. However, in the HPLC-APCI-MS

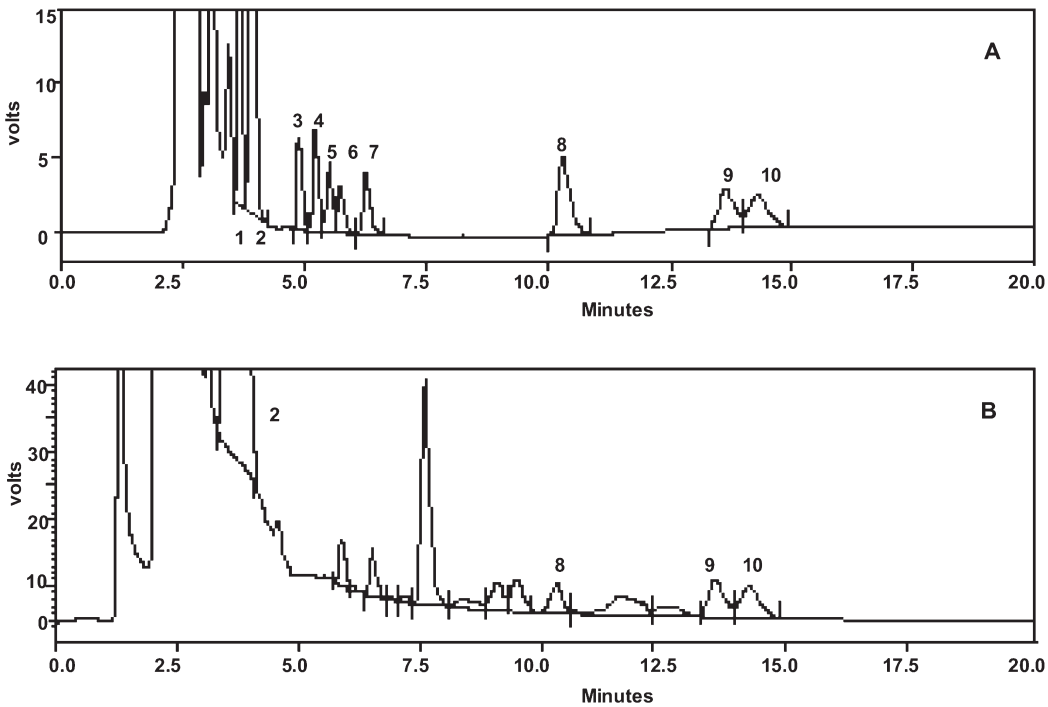

FIG. 1. Chromatograms of cholesterol and cholesterol oxides standards (A) and the commercial spray-dried whole egg powder (B), determined using HPLC-UV. The numbered peaks correspond to (1) 19-OH, (2) cholesterol, (3) 20α-OH, (4) 22(*R*)-OH, (5) 24(*S*)-OH, (6) 22(*S*)-OH, (7) 25-OH, (8) 7-keto, (9) 7β-OH, and (10) 7α-OH.

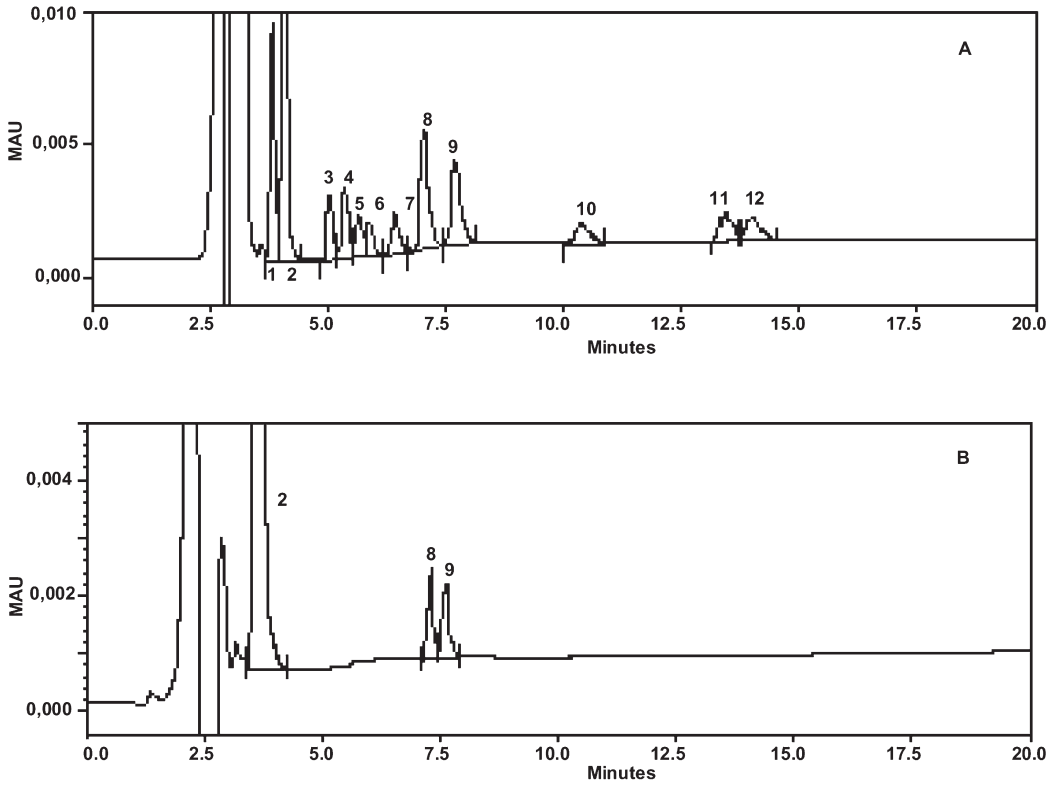

FIG. 2. Chromatograms of cholesterol and cholesterol oxides standards (A) and the commercial spray-dried whole egg powder (B), determined using HPLC-RI. The numbered peaks correspond to (1) 19-OH, (2) cholesterol, (3) 20α-OH, (4) 22(*R*)-OH, (5) 24(*S*)-OH, (6) 22(*S*)-OH, (7) 25-OH, (8) 5,6α-epoxy, (9) 5,6β-epoxy, (10) 7-keto, (11) 7β-OH, and (12) 7α-OH.

chromatograms of the same samples, the fragmentation patterns of the peaks observed with the same retention time as 20α-OH and 25-OH did not correspond to the patterns of these oxides.

The same five oxides were also found by Fontana *et al.* (22). In their study, when the powdered egg samples were heated at 90°C for 24 h, the formation of triol and 25-OH was observed, plus a product with a retention time similar to the 20α-OH, which was not confirmed by high-resolution NMR (22). In other studies the oxides trio, 25-OH and 20α-OH were not detected (23,24) or were not mentioned (25,26).

Characterization, quantification, and confirmation of cholesterol and cholesterol oxides by HPLC-APCI-MS. Table 1 presents the main molecular and fragment ions of cholesterol and its oxides produced in APCI(+)MS. Cholesterol, 19-OH, 25-OH, and 7-keto showed distinctive ion distributions, but 20α-OH, 22(*R*)-OH, 24(*S*)-OH, and 22(*S*)-OH are structurally very similar isomers, resulting in small differences in their ion distributions. Nevertheless, these differences, coupled with their characteristic retention times (the chromatographic peaks of these compounds were clearly resolved), were sufficient to confirm the identity of these oxides.

The 5,6α-epoxy and 5,6β-epoxy as well as 7α-OH and 7β-OH oxides also displayed similar fragmentation patterns, but these also have well-resolved chromatographic peaks, and could therefore be distinguished by their retention times. The triol oxide, which displayed similar fragmentation patterns to those of 5,6α-epoxy and 5,6β-epoxy, could be clearly distinguished from these oxides as it eluted at the end of the chromatographic run.

When analyzing the egg samples, the high cholesterol concentration made the recognition of the oxides difficult. This problem was avoided by removing the *m/z* 369 ion from the SIM run. Thus cholesterol could still be identified from its

TABLE 1
Relative Intensity of the Main Ions of Cholesterol and Cholesterol Oxides in HPLC-APCI(+)MS

	Relative intensity (m/z)				
Compounds[a]	367	385	369	401	403
19-OH	50	100	10	—	—
Cholesterol	5	5	100	—	—
20α-OH	100	95	10	—	—
22(*R*)-OH	100	100	10	—	—
24(*S*)-OH	100	100	10	—	—
22(*S*)-OH	85	100	10	—	—
25-OH	100	30	10	—	—
5,6α-Epoxy	50	100	10	—	20
5,6β-Epoxy	25	100	10	—	10
7-Keto	—	—	—	100	10
7β-OH	100	30	10	—	—
7α-OH	100	30	10	—	—
Triol	16	100	—	—	13

[a]Compounds are listed in order of elution.

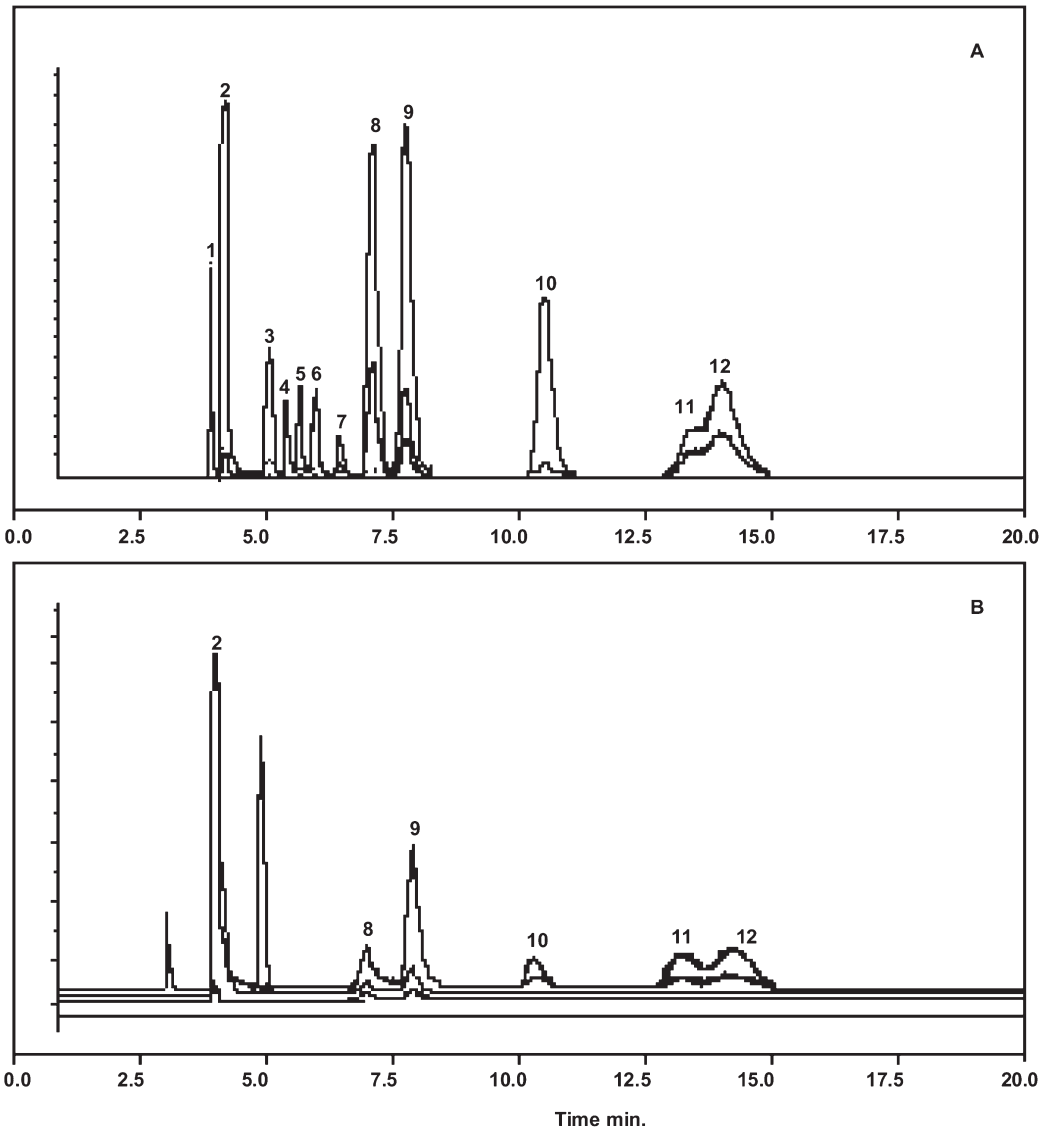

FIG. 3. Chromatograms of cholesterol and cholesterol oxides standards (A) and the commercial spray-dried whole egg powder (B), determined using HPLC-APCI-MS. The numbered peaks correspond to (1) 19-OH, (2) cholesterol, (3) 20α-OH, (4) 22(*R*)-OH, (5) 24(*S*)-OH, (6) 22(*S*)-OH, (7) 25-OH, (8) 5,6α-epoxy, (9) 5,6β-epoxy, (10) 7-keto, (11) 7β-OH, and (12) 7α-OH.

less intense fragment ions of *m/z* 367 and 385, and the peaks of the oxides could also be observed. Figure 3B shows the confirmation of the presence of cholesterol, 7-keto, 7β-OH, 7α-OH, 5,6α-epoxy, and 5,6β-epoxy and Figure 4B shows the absence of triol in the commercial spray-dried whole egg powder.

In addition to the identification of the cholesterol oxides, a comparison between the results obtained by HPLC-APCI-MS and HPLC-UV-RI was carried out. As can be seen in Table 2, the results obtained by different detectors showed no significant difference ($P > 0.05$), demonstrating that all these detector systems can be used to quantify cholesterol and the oxides found in this study. The methodology was validated using the HPLC-UV-RI system and was applied to determine the cholesterol oxides in egg samples.

Method validation. Recovery was carried out at two levels of addition; 50 and 100 μg for the oxides found in the samples, and 100 and 200 μg for cholesterol. Each level of addition was carried out in duplicate. Table 3 shows the mean values for the recovery of cholesterol and cholesterol oxides in commercial spray-dried whole egg powder. The method showed good results for recovery, from 89 to 103% for the oxides and from 93 to 96% for cholesterol. Studies using the direct saponification method gave a higher recovery value for cholesterol (27), a lower value for 7-keto, and similar values for the oxides 7β-OH and 7α-OH (15).

The low values found for DL and QL (Table 4) demonstrate the adequate sensitivity of the method used in the analyses of cholesterol and its oxides. Repeatability, verified from the relative standard deviations (RSD) values (Table 5), varied from

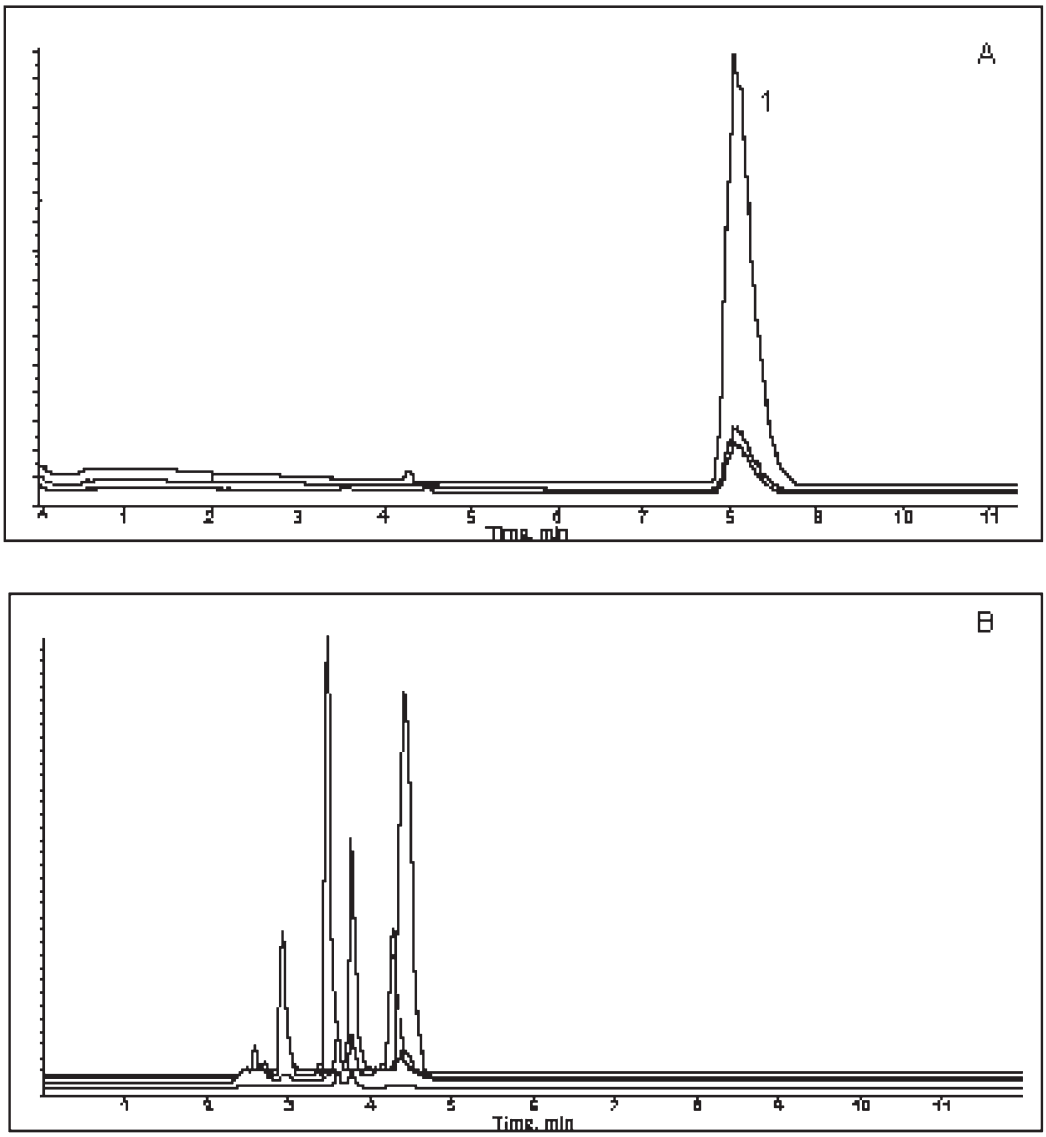

FIG. 4. Chromatograms of triol standard (A) and the commercial spray-dried whole egg powder (B), determined using HPLC-APCI-MS. The numbered peak corresponds to (1) triol.

4.3 to 15.2% for the oxides and from 2.5 to 3.7% for cholesterol. These values are considered acceptable for analytical repetitions (28), although Morgan and Armstrong (25) reported lower RSD values (3.1–8.0%) for the oxides.

The cholesterol content determined in the standard reference material (SRM 1845, NIST) was 19.0 ± 0.2 mg/g in

TABLE 2
Cholesterol Oxide Levels (mg/g) in Spray-Dried Whole Egg Powder by HPLC-UV-RI and HPLC-APCI-MS

Compounds	HPLC-UV-RI[a]	HPLC-APCI-MS[a]
7-Keto	0.34 ± 0.08[a]	0.35 ± 0.06[a]
7β-OH	32.87 ± 4.63[a]	32.83 ± 3.56[a]
7α-OH	24.64 ± 3.55[a]	23.97 ± 2.89[a]
5,6α-Epoxy	35.93 ± 5.90[a]	35.21 ± 2.80[a]
5,6β-Epoxy	51.24 ± 6.08[a]	50.88 ± 5.88[a]

[a]Results are reported as means ± estimated SD, $n = 10$. Means in the same row bearing same letters do not differ significantly ($P > 0.05$).

TABLE 3
Recovery (%) of Cholesterol and Cholesterol Oxides in Spray-Dried Whole Egg Powder

Compounds	Level of addition (μg)	Recovery (%)[a]
Cholesterol	100	93 ± 5
	200	96 ± 5
7-Keto	50	96 ± 5
	100	95 ± 4
7β-OH	50	97 ± 4
	100	92 ± 5
7α-OH	50	91 ± 5
	100	101 ± 8
5,6α-Epoxy	50	94 ± 4
	100	95 ± 5
5,6β-Epoxy	50	92 ± 4
	100	97 ± 6
Triol	50	89 ± 6
	100	103 ± 9

[a]Results are reported as mean and estimated SD of triplicate of 10 analyses.

TABLE 4
Detection and Quantification Limits for Cholesterol and Cholesterol Oxides

Compounds[a]	DL (ng/g)[b]	QL (ng/g)[b]
19-OH	31	42
Cholesterol	26	88
20α-OH	2	5
22(*R*)-OH	21	35
24(*S*)-OH	21	30
22(*S*)-OH	21	41
25-OH	2	6
5,6α-Epoxy	20	50
5,6β-Epoxy	20	64
7-Keto	2	6
7β-OH	25	79
7α-OH	10	62
Triol	31	65

[a]Compounds are listed in order of elution.
[b]Results are reported as the means of 10 analyses. DL, detection limit; QL, quantification limit.

whole egg powder, identical to the value declared on the certificate of analysis.

Application of methodology on eggs samples. Of the 12 oxides studied, the following 5 oxides were identified, quantified, and confirmed in commercial spray-dried whole egg powder: 7-keto, 7α-OH, 7β-OH, 5,6α-epoxy, and 5,6β-epoxy. The following three oxides were found in fresh eggs: 7-keto, 7α-OH, and 7β-OH. Only 7α-OH and 7β-OH were found in fresh eggs enriched with n-3 PUFA (Table 5). A great variety of values for the amounts of cholesterol oxides determined in spray-dried whole egg powder can be found in the literature, varying from not detected to 37 mg/g for 7-keto, from not detected to 65 mg/g for 7b-OH, from not detected to 35 mg/g for 7a-OH, from 12 to 111 mg/g for 5,6a-epoxy, and from 5 to 46 mg/g for 5,6b-epoxy (29,30). In the present study the valves obtained for all oxides were lower than the maximum described in literature. The discrepancies found in the amounts of the oxides are probably due to differences in the spray-drying processes used and in part due to the different methodologies used to determine the oxides.

Similar values were found by Van de Bovenkamp *et al.* (20) for 7-keto in fresh egg yolk. Nourooz-Zadeh (31) quantified 7-keto (2.2 mg/g of lipid) and 7a-OH (2.7 mg/g of lipid) in fresh eggs. On the other hand, none of the eight cholesterol oxides commonly found in foods were detected in fresh eggs (32).

The formation of high amounts of cholesterol oxides in eggs enriched with n-3 PUFA would be expected; however, in this case, the eggs enriched with n-3 PUFA presented only two oxides, and in smaller amounts than those found in fresh eggs. Probably, those results are due to the addition of antioxidants such as vitamin E to birds' diets rich in n-3 PUFA (33).

The mean values found for cholesterol were higher for samples of both egg and egg enriched with n-3 PUFA, and similar for whole egg powder, than those reported by the USDA (34).

In summary, the factors studied in the extraction design influenced the cholesterol and cholesterol oxide contents. It was possible to establish the best conditions for saponification and extraction of non-saponifiable matter using response surface methodology. The proposed method was shown to have high sensitivity, precision, and recovery and to be easy to apply in the separation of cholesterol and cholesterol oxides in eggs. Confirmation of the oxides 20α-OH and 25-OH is required, as the presence of these oxides was not confirmed by HPLC-APCI-MS.

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TABLE 5
Cholesterol and Cholesterol Oxides in Eggs

	Spray-dried whole egg powder		Fresh egg yolk		Fresh egg yolk enriched with n-3 PUFA	
Compounds	Mean ± SD[a]	RSD (%)	Mean ± SD[a]	RSD (%)	Mean ± SD[a]	RSD (%)
Cholesterol[b]	1,715 ± 63	3.7	2,481 ± 62	2.5	2,401 ± 68	2.8
7-Keto[c]	2.50 ± 0.38	15.2	0.36 ± 0.02	6.3	ND	—
7α-OH[c]	5.18 ± 0.50	9.6	15.90 ± 1.12	7.0	3.88 ± 0.19	5.0
7β-OH[c]	6.17 ± 0.88	14.2	3.73 ± 0.19	5.1	3.02 ± 0.13	4.3
5,6α-Epoxy[c]	9.73 ± 1.09	11.3	ND	—	ND	—
5,6β-Epoxy[c]	17.63 ± 1.87	10.6	ND	—	ND	—

[a]Mean and estimated SD are of triplicates. ND, not detected (see Table 3 for detection limits); RSD = relative standard deviation.
[b]Values are in mg/100 g, dry weight basis.
[c]Values are in mg/g, dry weight basis.

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COMMUNICATION

Identification and Production of n-8 Odd-Numbered Polyunsaturated Fatty Acids by a Δ12 Desaturation-Defective Mutant of *Mortierella alpina* 1S-4

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ABSTRACT: A mutant of *Mortierella alpina*, JT-180, which is defective in its Δ12 desaturase activity but exhibits enhanced activities of Δ5 and Δ6 desaturases, produced a high proportion (up to 80%) of odd-chain FA when grown on 3% *n*-heptadecane. Following growth of the mutant on *n*-heptadecane, three unusual odd-chain fatty acyl residues were identified as 6,9-heptadecadienoic acid (17:2), 8,11-nonadecadienoic acid (19:2), and 5,8,11-nonadecatrienoic acid (19:3) by means of GC-MS, MS-MS, and NMR analyses. The mycelial contents of these FA reached 20.3, 3.6, and 5.8 mg/g dry mycelia, respectively, when it was cultivated in medium comprising 4% (vol/vol) *n*-heptadecane and 1% (wt/vol) yeast extract, pH 6.0, at 28°C for 7 d. The biosynthetic route (n-8 route) to 19:3 was presumed to mimic the n-9 route to Mead acid (20:3n-9) in mammals: 17:0 → 9-17:1 → 6,9-17:2 → 8,11-19:2 → 5,8,11-19:3.

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The occurrence of odd-numbered, straight-chained PUFA has been reported in ruminants, fish, and several other animal lipids, in which they can account for a small percentage of the total FA. Moreover, such PUFA can undergo further desaturation reactions; for example, 9,12-heptadecadienoic acid and 6,9,12-heptadecatrienoic acid prepared from mullet oil are converted to odd-chain PUFA such as 8,11,14-nonadecatrienoic acid and 5,8,11,14-nonadecatetraenoic acid (19:4) in rat liver (1). In microorganisms, odd-chain-length FA may be produced when yeasts, bacteria, and fungi are grown on odd-chain-length alkanes (2–4). Additionally, several *Mortierella* species (5) can accumulate C_{19} PUFA derived from methyl pentadecanoate or *n*-pentadecane added to the medium. In particular, on cultivation of the arachidonic acid–producing *Mortierella alpina* 1S-4 with 5% *n*-heptadecane as the growth substrate, the amount of 19:4 reached 44.4 mg/g dry mycelia (0.7 mg/mL of culture broth). Here we report that a mutant of this fungus, JT-180, which is defective in its Δ12 desaturase activity but exhibits enhanced activities of Δ5 and Δ6 desaturases, can accumulate large amounts of unusual C_{17} and C_{19} PUFA in its mycelial lipids when grown with odd-chain *n*-alkanes. Normally when this mutant is grown on glucose or similar substrates, it produces Mead acid (20:3n-9) as its major PUFA (6).

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MATERIALS AND METHODS

Chemicals. Odd-chain *n*-alkanes were purchased from Wako Pure Chemicals (Osaka, Japan). All reagents were of analytical grade.

Microorganism and cultivation. Strain JT-180, which is a mutant derived from *M. alpina* 1S-4 by chemical mutagenesis (7), was inoculated as a spore suspension into 20-mL Erlenmeyer flasks containing 4 mL of medium containing 3% (vol/vol) *n*-alkane and 1% (wt/vol) yeast extract (pH 6.0). It was grown at 28°C with reciprocal shaking (120 strokes/min) unless stated otherwise.

FA analysis. The cultivated mycelia were harvested by vacuum filtration, washed rapidly with *n*-hexane to remove residual *n*-alkanes from the cell surface, dried at 120°C for 3 h, and then transmethylated with methylene chloride/10% methanolic HCl (vol/vol) at 50°C for 3 h. The resultant FAME were extracted with *n*-hexane and then analyzed by GC, as described previously (8).

Identification. The FAME derived from JT-180 cultivated in a medium containing 2% (wt/vol) glucose, 1% (wt/vol) yeast extract, and 1% (vol/vol) *n*-heptadecane, pH 6.0, at 28°C for 10 d were first separated by HPLC using a Cosmosil column (5C18-AR, 20 × 250 mm, Nacalai Tesque, Kyoto, Japan). Acetonitrile/H_2O (8:2, vol/vol) was used as the mobile phase at 4 mL/min, and the effluent was monitored at 205 nm. The semipurified FAME were completely separated by HPLC on a different column (Cosmosil column 5C18-AR, 4.6 × 250 mm). The isocratic mobile phase was acetonitrile/H_2O (9:1, vol/vol) at 3 mL/min. The chemical structures of these FAME were determined by MS-MS and ^1H NMR. The MS-MS analyses were performed with a JEOL-HX110A/HX110A tandem mass spectrometer. The ionization method was FABMS, and the acceleration voltage was 3 kV. Glycerol was used as the matrix. The NMR experiment was performed with a Bruker Biospin DMX-750 (750 MHz for ^1H), and chemical shifts were assigned relative to the solvent signal. FAME were dissolved in $CDCl_3$ and analyzed by the two-dimensional NMR technique of ^1H-^1H chemical shift COSY.

GC-MS analysis of pyrrolidide derivatives. The purified FAME were converted to pyrrolidide derivatives as described previously (9). The pyrrolidide derivatives were identified by GC-MS using a Shimadzu GCMS-QP5050 operating at an ionization voltage of 70 eV.

RESULTS

Identification of the unknown odd-chain FA. When the mutant, JT-180, was cultivated in the medium containing 1% *n*-heptadecane, it accumulated three unknown FA (UK1, UK2, and UK3). The mass spectrum of the pyrrolidide derivatives of UK1, UK2, and UK3 showed molecular weights of *m/z* 319, 345, and 347, respectively. These results suggested that UK1, UK2, and UK3 were, respectively, a C_{17} FA with two double bonds, a C_{19} FA with three double bonds, and a C_{19} FA with two double bonds. The FABMS data for the free FA of UK1 indicated a molecular weight of 266 (Li-complex, *m/z* 287 $[M + Li]^+$). The material (*m/z* 287) was fragmented again by MS-MS, yielding *m/z* (FABMS$^-$, 8.00 kV) peaks at 271 (1.2), 257 (1.2), 243 (3.1), 215 (10.5), 201 (6.8), 187 (1.5), 175 (13.2), 161 (17.6), 147 (5.4), 135 (1.0), 121 (2.3), 107 (10.1), 94 (17.9), 93 (38.7), 80 (100), 79 (9.4), and 51 (8.7). The *m/z* 121, 147, 161, and 187 fragments were derived from cleavage between single bands 6-7 and 9-10, numbered from the carboxyl group. ^{1}H NMR analysis also revealed that UK1 was a 17:2 isomer, with NMR δ_H ($CDCl_3$) at 5.38 (4H, *m*, CH_2-C<u>H</u>=C<u>H</u>-CH_2), 3.67 (3H, *s*, O-C<u>H</u>$_3$), 2.77 (2H, *dd*, *J* = 6.14 Hz, =CH-C<u>H</u>$_2$-CH=), 2.21 (2H, *t*, *J* = 7.54 Hz, O=C-C<u>H</u>$_2$), 2.06 (4H, *dt*, *J* = 6.80, 2.60 Hz, CH_2-C<u>H</u>$_2$-CH=), 1.62 (2H, *tt*, *J* = 7.77, 7.58 Hz, CH_2-C<u>H</u>$_2$-CH_2), 1.39 (2H, *tt*, *J* = 7.71, 7.75 Hz, CH_2-C<u>H</u>$_2$-CH_2), 1.36 (10H, *m*, CH_2-C<u>H</u>$_2$-CH_2), 0.97 (3H, *t*, CH_2-C<u>H</u>$_3$). The sequence of the protons was determined on the basis of the signal pattern of the interactions between adjacent protons observed on COSY (data not shown). On the basis of these results, UK1 was identified as 6,9-heptadecadienoic acid (17:2n-8).

The FABMS data for the free FA of UK2 indicated a molecular weight of 292 (Li-complex, *m/z* 313 $[M+Li]^+$). The material (*m/z* 313) was fragmented again by MS-MS, yielding *m/z* (FABMS$^-$, 8.00 kV) peaks at 297 (1.2), 283 (5.7), 269 (4.6), 255 (4.0), 241 (17.5), 227 (22.9), 213 (3.6), 201(11.4), 187 (54.2), 174 (4.3), 161(9.0), 147 (22.3), 134 (2.23), 133(6.7), 121 (1.1), 103(12.0), 94 (100.0), 93 (75.6), 80 (86.3), and 50 (30.1). The *m/z* 121, 147, 161, 187, 201, and 227 fragments were derived from cleavage between single bands 5-6, 8-9, and 11-12, numbered from the carboxyl group. ^{1}H NMR analysis also revealed that UK2 was a 19:3 isomer, with NMR δ_H ($CDCl_3$) at 5.38 (6H, *m*, CH_2-C<u>H</u>=C<u>H</u>=C<u>H</u>-CH_2), 4.22 (3H, *s*, O-C<u>H</u>$_3$), 2.80 (4H, *dd*, *J* = 7.02 Hz, =CH-C<u>H</u>$_2$-CH=), 2.33 (2H, *t*, *J* = 7.54 Hz O=C-C<u>H</u>$_2$), 2.12 (2H, *dt*, *J* = 7.25 Hz, =CH-C<u>H</u>$_2$-CH_2), 2.05 (2H, *dt*, *J* = 7.16, 7.35 Hz, =CH-C<u>H</u>$_2$-CH_2), 1.71 (2H, *tt*, *J* = 6.55, 5.78 Hz, CH_2-C<u>H</u>$_2$-CH_2), 1.36 (10H, *m*, CH_2-C<u>H</u>$_2$-CH_2), 0.93 (3H, *t*, *J* = 7.51 Hz, CH_2-C<u>H</u>$_3$). The sequence of the protons was determined on the basis of the signal pattern of the interactions between adjacent protons observed on COSY (data not shown). On the basis of these results, UK2 was identified as 5,8,11-nonadecatrienoic acid (19:3n-8).

The pyrrolidide derivative of UK3 was fragmented by GC-MS, yielding peaks at *m/z* 347 (7.1), 318 (0.4), 304 (0.5), 290 (0.4), 276 (2.1), 262 (0.8), 248 (0.9), 222 (2.5), 208 (6.2), 194 (2.1), 182 (2.9), 168 (7.5), 154 (3.3), 140 (5.8), 126 (53.2), and 113 (100.0). The spectra included significant *m/z* 113 and 126 peaks typical of FA pyrrolidide derivatives. The differences of 26 atom mass units between *m/z* 248 and 222 and *m/z* 208 and 182 each indicated a double bond. As a result, UK3 was identified as 8,11-nonadecadienoic acid (19:2n-8).

Conversion of odd-chain alkanes to odd-chain FA by JT-180. The mycelia of JT-180 grown in the medium containing 3% (vol/vol) *n*-alkane and 1% (wt/vol) yeast extract accumulated considerable amounts of unusual odd-chain FA, as shown in Tables 1 and 2. Among these FA, three major components showed the same retention times as authentic 15:0, 17:0, and 19:0 FAME on GC and HPLC analyses. The structures of the other unknown FA were determined by the analytical methods described in the previous section. When JT-180 was cultivated in a medium containing 2% (wt/vol) glucose, 1% (wt/vol) yeast extract, and 1% (vol/vol) *n*-alkane, the added *n*-alkanes did not inhibit fungal growth and, indeed, its dry mass was higher than that without *n*-alkane (data not shown). Both the C_{15} and C_{17} alkanes were efficiently converted to C_{17} and C_{19} PUFA. In particular, with *n*-heptadecane, the total odd-chain FA accounted for almost 80% of the total FA (see Table 1).

Furthermore, the mycelial mass and the sum of 17:2, 19:2, and 19:3 yields were highest, 21.3 mg/mL and 28.8 (15.8, 5.1,

TABLE 1
Comparison of FA Compositions of JT-180 Grown with Odd-Chain *n*-Alkanes[a]

FA added	FA composition (%)													
	15:0	17:0	17:1	17:2	19:0	19:1	19:2	19:3	16:0	18:1	18:2	20:3	Others[b]	Total odd-chain FA
None	0.9 ± 0.1	3.1 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	1.5 ± 0.2	0.2 ± 0.1	3.5 ± 0.3	3.6 ± 0.2	40.9 ± 0.5	13.4 ± 0.9	23.2 ± 0.8	7.9 ± 0.2	11.1 ± 0.8
C_{11}	5.2 ± 0.1	4.1 ± 0.5	2.7 ± 0.2	2.1 ± 0.2	0.7 ± 0.1	1.8 ± 0.2	0.8 ± 0.1	5.2 ± 0.1	7.8 ± 0.5	40.3 ± 0.3	9.0 ± 0.7	11.6 ± 0.7	8.7 ± 0.1	22.6 ± 0.4
C_{13}	13.5 ± 0.2	10.2 ± 0.2	9.8 ± 0.2	7.8 ± 0.2	0.6 ± 0.1	5.6 ± 0.2	3.4 ± 0.0	8.4 ± 0.1	4.0 ± 0.2	24.4 ± 0.4	4.2 ± 0.2	3.4 ± 0.1	4.7 ± 0.1	59.3 ± 0.5
C_{15}	25.5 ± 0.2	10.5 ± 0.2	15.0 ± 0.1	10.5 ± 0.4	0.6 ± 0.0	5.8 ± 0.1	3.7 ± 0.1	7.0 ± 0.1	1.8 ± 0.1	14.5 ± 0.4	2.0 ± 0.0	1.5 ± 0.2	1.6 ± 0.1	78.5 ± 0.8
C_{17}	9.9 ± 0.3	23.6 ± 0.6	19.3 ± 0.9	10.7 ± 0.7	0.8 ± 0.3	5.9 ± 0.2	3.4 ± 0.1	5.2 ± 0.3	2.5 ± 0.2	13.1 ± 0.8	1.9 ± 0.1	1.6 ± 0.2	2.0 ± 0.6	78.8 ± 1.8
C_{19}	3.6 ± 0.3	6.5 ± 0.3	4.9 ± 0.3	3.2 ± 0.2	1.3 ± 0.1	9.3 ± 0.4	1.1 ± 0.0	5.7 ± 0.2	3.5 ± 0.3	32.6 ± 0.9	8.5 ± 0.1	14.2 ± 0.2	5.7 ± 0.3	35.5 ± 1.4

[a]JT-180 was grown in medium containing 1% (wt/vol) yeast extract and 3% (vol/vol) *n*-alkane, pH 6.0, at 28°C, for 7 d. Values are means ± SD, *n* = 3. C_{11}, *n*-undecane; C_{13}, *n*-tridecane; C_{15}, *n*-pentadecane; C_{17}, *n*-heptadecane; C_{19}, *n*-nonadecane; 15:0, petadecanoic acid; 17:0, heptadecanoic acid; 17:1, 9-heptadecenoic acid; 17:2, 6,9-heptadecadienoic acid; 19:0, n-nonadecanoic acid; 19:1,11-nonadecenoic acid; 19:2, 8,11-nonadecadienoic acid; 19:3, 5,8,11-nonadecatrienoic acid; 16:0, palmitic acid,; 18:1, oleic acid; 18:2, 6,9-octadecadienoic acid; 20:3, Mead acid.
[b]Others include 16:1 (palmitoleic acid), 18:0 (stearic acid), 20:0 (eicosanoic acid), 20:1 (11-eicosaenoic acid), 20:2 (8,11-eicosadienoic acid), 22:0 (docosanoic acid), and 24:0 (tetracosanoic acid).

and 7.9) mg/g dry mycelia, respectively, with *n*-heptadecane (see Table 2). On the other hand, the use of C_{13} alkane brought about the highest yield (8.4%) and mycelial content (12.8 mg/g dry mycelia) of 19:3, whereas the mycelial mass was not higher than those in the use of C_{15} or C_{17} alkanes. Shorter and longer carbon chain alkanes (*i.e.,* C_{11} and C_{19}) were not efficiently converted. When JT-180 was grown with C_{15}, C_{17}, and C_{19} saturated FAME, the C_{17} and C_{19} PUFA were not accumulated with the same efficiency as with the corresponding *n*-alkanes (data not shown).

Effects of n-heptadecane concentration and cultivation temperature on production of odd-chain PUFA. JT-180 was cultivated in medium containing 1% (wt/vol) yeast extract and 3, 4, or 5% (vol/vol) *n*-heptadecane at 12, 20, or 28°C, and the mycelial FA contents were analyzed as shown in Table 3. The mycelial contents of 17:2 and 19:2 were highest at the cultivation temperature of 28°C independent of the *n*-heptadecane concentration, whereas that of 19:3 was highest only at 20°C. On the other hand, the culture condition with 4% (vol/vol) *n*-heptadecane at 28°C led to highest mycelial mass of 21.6 mg/mL, and the mycelial contents of 17:2, 19:2, and 19:3 reached 20.3, 3.6, and 5.8 mg/g dry mycelia, respectively. The sum of the mycelial contents of these FA represented the maximal value at 28°C. The conversion rates ([produced 19:3/added *n*-heptadecane] × 100) were 0.41, 0.31, and 0.22 with 3, 4, and 5% (vol/vol) of *n*-heptadecane, respectively. As a result, the conversion rate was higher at the lower concentration of *n*-heptadecane.

DISCUSSION

Shimizu *et al.* reported that some *Mortierella* species, such as *M. alpina*, *M. elongata*, *M. verticillata*, and *M. kuhlmanii*, which normally produce C_{20} PUFA, could accumulate some C_{17} and C_{19} FA when grown with odd-chain *n*-alkanes (5). In particular, an arachidonic acid–producing fungus, *M. alpina* 1S-4, accumulated a large amount (44.4 mg/g dry mycelia) of 19:4 corresponding to arachidonic acid minus one carbon unit at the ω-terminal end (5). One of the predominant C_{19} PUFA produced by Mead acid–producing JT-180 (defective in Δ12 desaturase activity but with enhanced activities of Δ5 and Δ6 desaturases) was identified as 19:3 corresponding to Mead acid minus one carbon unit at the w-terminal end. The other odd-chain PUFA formed by JT-180 were identified as 17:2 and 19:2. These data suggest that these odd-chain PUFA were biosynthesized through the proposed pathway in Figure 1, which mimics the biosynthetic pathway for the n-9 series, in which the following are the successive reactions involved: oxidation of *n*-heptadecane to 17:0, desaturation to 6,9-17:2 via 9-17:1, elongation of 17:2 to 8,11-19:2, and further desaturation of 19:2 to 5,8,11-19:3. These odd-chain PUFA correspond to the equivalent even-chain PUFA minus one carbon unit at the ω-terminal end. The wild strain, *M. alpina* 1S-4, could not produce detectable amounts of n-8 series odd-chain PUFA such as 17:2, 19:2, and 19:3 because of its own Δ12 desaturation. On the other hand, in mammalian cells lacking Δ12-desaturation activity, n-8 series odd-chain PUFA may be inefficiently derived from exogenous or endogenous odd-chain FA.

TABLE 2
Production of Odd-Chain PUFA by JT-180 Grown with *n*-Alkanes[a]

FA added	Mycelial mass (mg/mL culture broth)	Mycelial FA content (mg/g dry mycelia)			
		17:2	19:2	19:3	MA
None	2.2 ± 0.2	0.4 ± 0.0	0.1 ± 0.0	1.7 ± 0.1	11.6 ± 0.1
C_{11}	5.7 ± 0.1	2.3 ± 0.2	0.9 ± 0.1	5.7 ± 0.4	12.7 ± 1.2
C_{13}	9.2 ± 0.6	11.9 ± 0.3	5.3 ± 0.1	12.8 ± 0.3	5.1 ± 0.1
C_{15}	18.5 ± 1.1	14.4 ± 1.1	5.1 ± 0.4	9.6 ± 0.2	2.0 ±0.1
C_{17}	21.3 ± 1.4	15.8 ± 1.0	5.1 ± 0.7	7.9 ± 1.2	2.5 ± 0.6
C_{19}	2.1 ± 0.5	3.1 ± 0.9	1.1 ± 0.3	5.6 ± 1.4	13.7 ± 3.1

[a]JT-180 was grown in medium containing 1% (wt/vol) yeast extract and 3% (vol/vol) *n*-alkane, pH 6.0, at 28°C, for 7 d. Values are means ± SD, $n = 3$.

TABLE 3
Effects of *n*-Heptadecane Concentration and Cultivation Temperature on Production of Odd-Chain PUFA in JT-180[a]

C_{17} (%)	Temp. (°C)	Mycelial mass (mg/mL)	Mycelial FA content (mg/g dry mycelia)			
			17:2	19:2	19:3	MA
3	12	10.5 ± 1.3	11.3 ± 1.8	2.0 ± 0.2	7.8 ± 0.6	1.8 ± 1.2
	20	10.5 ± 3.1	9.2 ± 1.5	1.7 ± 0.2	9.4 ± 1.6	2.2 ± 1.1
	28	19.3 ± 0.6	20.3 ± 1.8	3.8 ± 0.4	6.4 ± 0.5	2.4 ± 0.2
4	12	14.4 ± 1.7	8.5 ± 2.7	1.5 ± 0.4	5.5 ± 1.0	1.4 ± 0.9
	20	10.2 ± 0.6	9.7 ± 0.3	1.7 ± 0.0	10.1 ± 0.4	2.1 ± 0.0
	28	21.6 ± 2.2	20.3 ± 1.4	3.6 ± 0.1	5.8 ± 0.4	2.4 ± 0.1
5	12	11.7 ± 3.5	9.6 ± 1.6	1.8 ± 0.3	8.1 ± 1.8	2.1 ± 0.7
	20	11.2 ± 1.3	9.9 ± 3.7	2.1 ± 0.7	8.9 ± 2.8	2.0 ± 0.7
	28	18.5 ± 1.2	19.3 ± 3.4	3.6 ± 0.9	6.0 ± 1.2	3.0 ± 1.1

[a]JT-180 was grown in medium containing 1% (wt/vol) yeast extract and 3, 4, or 5% (vol/vol) *n*-heptadecane, pH 6.0, at 28°C for 2 d and then at 12, 20, or 28°C for 5 d. Values are means ± SD, $n = 3$.

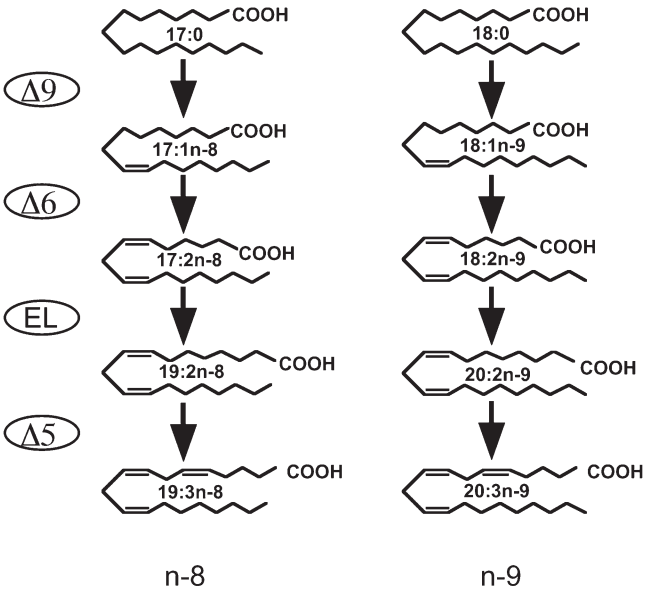

FIG. 1. Putative biosynthetic pathway for n-8 series odd-chain PUFA in JT-180. ΔX, ΔX FA desaturation; EL, FA elongation.

The 15:0 and 17:0 FAME were not effectively converted to 19:3, compared with *n*-alkanes as substrates (data not shown). The C_{11} or C_{19} alkanes were also not effectively converted to 19:3. The uptake efficiencies of fatty substrates into mycelia, oxidation of *n*-alkanes into FA, and elongation of shorter-chain FA into C_{19} FA may influence the rate of conversion to 19:3. *n*-Heptadecane as a sole carbon source led to the highest mycelial growth and total odd-chain FA yield. This indicates that JT-180 efficiently uptakes *n*-heptadecane and converts it to FA. In contrast, although C_{13} alkane led to the highest rate and mycelial content of 19:3, the mycelial mass and FA yield were not higher. This suggests that uptake efficiency of C_{13} alkane into mycelia is very low; however, the C_{13} alkane is easily converted to 19:3 once incorporated. Regardless of *n*-heptadecane concentration, the mycelial content of 19:3 showed the maximal value of 8.9–10.1 mg/g dry mycelia at the cultivation temperature of 20°C as shown in Table 3. It is assumed that FA elongation (17:2 → 19:2) and Δ5 desaturation (19:2 → 19:3) were activated at 20°C, resulting in more accumulation of 19:3 within mycelia. Previously, the highest accumulation of Mead acid was observed for JT-180 at the same cultivation temperature (6). This may be one reason why Δ5 and Δ6 desaturases show high activities at the lower cultivation temperature of 20°C. The low FA yield as shown in Table 3 may be due to the solid state of *n*-heptadecane at 12°C, which prevents incorporation of *n*-heptadecane into mycelia. Just considering the conversion rate of 19:3, 3% (vol/vol) of *n*-heptadecane may be comparable to the optimal concentration. Excess added *n*-heptadecane is thought to remain in the medium or adhere to the cell surface.

FAME containing mainly n-5 series odd-chain PUFA, such as 9,12-heptadecadienoic acid and 6,9,12-heptadecatrienoic acid, have been shown to cure the external symptoms of fat-deficient rats through conversion to 19:4 in the rat liver (1). However, other physiological functions of odd-chain PUFA have not been fully elucidated yet. When JT-180 was cultivated with 4% (vol/vol) *n*-heptadecane in 28°C for 7 d, the total amounts of 17:2, 19:2, and 19:3 reached 29.7 mg/g dry mycelia (see Table 3). n-8 Series odd-chain PUFA are unusual in natural sources, and this study suggests a new source of three odd-chain PUFA. We expect that n-8 series odd-chain PUFA should be useful as chemical reagents or help to clarify their own physiological functions in mammals.

ACKNOWLEDGMENTS

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Acyl Moieties Modulate the Effects of Phospholipids on β-Carotene Uptake by Caco-2 Cells

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ABSTRACT: The intestinal absorption of carotenoids is thought to be mediated by the carotenoid assembly in mixed micelles, followed by its transfer into the enterocytes and subsequent secretion to the lymph as chylomicron particles. In the present study we investigated the effects of phospholipids and lysophospholipids with diverse fatty acyl moieties on the uptake of β-carotene solubilized in mixed micelles by Caco-2 cells. Compared with phospholipid-free mixed micelles (NoPL), those containing long-chain PC inhibited β-carotene uptake (16:0,18:1-PC ≅ 16:0,18:2-PC < 14:0,14:0-PC ≅ 16:0,14:0-PC < 16:0,16:0-PC < NoPL). However, mixed micelles containing medium-chain PC enhanced β-carotene uptake (NoPL < 8:0,8:0-PC < 12:0,12:0-PC < 10:0,10:0-PC), and short-chain PC did not affect the uptake. Among the lysophosphatidylcholine (LysoPC) class, a marked increase of β-carotene uptake by medium-to-long-chain LysoPC was observed (NoPL < 12:0-LysoPC < 14:0-LysoPC < 18:1-LysoPC < 16:0-LysoPC), although short-to-medium-chain LysoPC (6:0-LysoPC to 10:0-LysoPC) did not affect β-carotene uptake. The long-chain 16:0,18:1-PC increased the β-carotene efflux from cells and drastically changed the β-carotene UV-visible absorbance spectrum, compared with those of NoPL micelles. The acyl moieties of long-chain PC may interact with the carotenoid in the micelle interior, shifting the β-carotene partition toward the micellar phase. Medium-chain PC and long-chain LysoPC, which have nearly equivalent hydrophobicities, may enhance β-carotene uptake through their interaction with the cell membrane.

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The intestinal absorption of dietary carotenoids comprises several steps, including the release of carotenoids from the food matrix, dispersion as lipid emulsion particles and assembly into mixed micelles in the digestive tract, uptake by intestinal epithelial cells, and secretion to lymph as chylomicrons (1,2). The uptake of carotenoids by the intestinal cells has been thought to occur through simple diffusion (3,4), similar to many other dietary lipids. However, recent observations indicate the existence of carotenoid transport mediated by the scavenger receptor class B type I (SR-BI) and possibly other receptors (5–7). The SR-BI–mediated transport seems to occur without energy expenditure, which implies the necessity of a concentration gradient between micelles and cells (8). Thus, an efficient solubilization of carotenoids into mixed micelles and release to cells would favor carotenoid uptake, whether it is mediated by simple diffusion or by facilitated transport.

Dietary and biliary lipids, together with their lipolytic products, are essential for the emulsification and micellar solubilization of carotenoids. Phospholipids, especially PC, are present in the diet either as a natural component of the food matrix or as an emulsifier/stabilizer in processed foods (9,10). Additionally, the bile constitutes a large physiological pool of phospholipids (mainly PC), which are introduced into the duodenum during the digestive process (11). In both foodstuff and bile, PC functions as an emulsifier and prevents the precipitation of lipophilic compounds. However, in contrast with the general idea that lipid absorption is proportional to its micellar solubilization, there has been some evidence indicating that PC suppresses the absorption of carotenoids and other lipophilic compounds despite promoting their emulsification and/or solubilization (12–17).

Our previous studies showed that PC inhibits the absorption of carotenoids by human intestinal Caco-2 cells (17) and mice (12). However, the replacement of PC by lysophosphatidylcholine (LysoPC) in micelles or the PC hydrolysis by phospholipase A_2 greatly improves β-carotene and lutein absorption by Caco-2 cells and experimental animals (12,17,18). Previous studies suggested that the acyl moieties of phospholipids interact with the lipophilic compounds in the micelle core, inhibiting their release for the uptake by enterocytes (13–16). The elimination of one acyl chain from PC, producing LysoPC, suggests a reduced hydrophobicity and a weaker association between the phospholipid and carotenoids in micelles, which is in accordance with the increase of carotenoid absorption observed by Sugawara *et al.* (17). However, the mechanisms underlying the effects of phospholipids on the intestinal uptake of carotenoids have not been fully elucidated, although the amphiphilic properties of phospholipids might affect the solubilization of carotenoids and cell membrane permeability. Because the acyl chain length of phospholipids is one of the critical factors determining their amphiphilic properties, it is worth evaluating the effects of phospholipids with a variety of fatty acyl moieties on carotenoid uptake by Caco-2 cells, in order to elucidate the mechanism of action of phospholipids on the intestinal absorption of carotenoids.

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Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium, FWHM, full width at half maximum; LysoPC, lysophosphatidylcholine; NoPL, phospholipid-free; SR-BI, scavenger receptor class B type I; TEER, transepithelial electrical resistance.

In the present study, we investigated the interactions between phospholipids and β-carotene in mixed micelles, using phospholipids and lysophospholipids with a wide range of acyl chains, and focusing on their resulting effects on β-carotene uptake by Caco-2 human intestinal cells.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise stated, all reagents for the preparation of micelles were purchased from Sigma-Aldrich Co. (St. Louis, MO). The effects of phospholipids and lysophospholipids on β-carotene uptake were assessed by using compounds with a wide range of acyl chain lengths. The effects of the acyl chain length and unsaturation of PC were evaluated by using 1,2-diacyl-*sn*-glycero-3-phosphocholine with the following fatty acyl groups: 1,2-dibutyryl (4:0,4:0-PC), 1,2-dihexanoyl (6:0,6:0-PC), 1,2-dioctanoyl (8:0,8:0-PC, Avanti Polar Lipids, Alabaster, AL), 1,2-didecanoyl (10:0, 10:0-PC), 1,2-didodecanoyl (12:0,12:0-PC), 1,2-dimyristoyl (14:0,14:0-PC), 1-palmitoyl-2-myristoyl (16:0,14:0-PC), 1,2-dipalmitoyl (16:0,16:0-PC), 1-palmitoyl-2-oleoyl (16:0,18:1-PC), and 1-palmitoyl-2-linoleoyl (16:0,18:2-PC). LysoPC acyl chain length and unsaturation effects were assessed by using 1-acyl-2-hydroxy-*sn*-glycero-3-phosphocholine with various acyl groups: hexanoyl (6:0-LysoPC), octanoyl (8:0-LysoPC, Avanti Polar Lipids), decanoyl (10:0-LysoPC, Avanti Polar Lipids), dodecanoyl (12:0-LysoPC), myristoyl (14:0-LysoPC), palmitoyl (16:0-LysoPC), and oleoyl (18:1-LysoPC). β-carotene (Type II, synthetic, ≥ 95% HPLC, crystalline) was dissolved in hexane and passed through a small column packed with 1.5 g of neutral alumina (grade III, ICN Biomedicals, Eschweger, Germany) in order to remove polar oxidation products. The eluate was stored at −80°C until use. All procedures involving β-carotene were performed under dim yellow light in order to minimize its degradation.

Cell culture. Caco-2 cells (American Type Culture Collection, Rockville, MD) were routinely maintained in 10-cm dishes, as previously reported (17). For the uptake and efflux experiments, the Caco-2 cells were seeded into 24-well plates at 10^5 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS), 4 mmol/L L-glutamine, 40,000 units/L penicillin, 40 mg/L streptomycin, and 0.1 mmol/L nonessential amino acids. The cells were cultured for 20–22 d at 37°C in a humidified atmosphere of 95% air and 5% CO_2, with the culture medium replaced every 2–3 d. For transport experiments, cells were seeded in polycarbonate Transwell® 6-well plates (Corning Inc., Corning, NY) at 7.5×10^5 cells/well, and cultured for 25–30 d under the same conditions described for uptake and efflux experiments. Transepithelial electrical resistance (TEER) was measured at the end of the culture period using a voltohmmeter equipped with a chopstick-type electrode (EVOMX and STX2 electrode, World Precision Instruments, Sarasota, FL); values of approximately 250 Ω were obtained, indicating the formation of tight monolayers.

Preparation of mixed micelles containing β-carotene. Mixed micelles were prepared as previously published (17). In brief, all components were previously dissolved in an adequate solvent, and appropriate volumes of each solution were transferred to glass test tubes. The solvent was evaporated under a stream of argon, and the samples were further submitted to vacuum centrifugation. The dry matter was vortexed with serum-free DMEM and filtered through a 0.20-μm PTFE membrane (DISMIC-25cs, Advantec Co., Tokyo, Japan). Each micellar component was added in the amount needed to reach the following concentrations: 2 mmol/L sodium taurocholate, 33.3 μmol/L oleic acid, 100 μmol/L monoolein, 1.0 μmol/L β-carotene, and 0 or 50 μmol/L of the phospholipid to be tested. The β-carotene concentration in the mixed micelles was set to 1.0 μmol/L in order to enable comparisons with our previous studies (17,19). This value is also comparable to the estimated β-carotene concentration in the micellar phase of the duodenal contents after the ingestion of a physiological dose of β-carotene from vegetables (20). The β-carotene concentration in the micelle preparations was determined by HPLC after a 9-fold dilution with dichloromethane/methanol (1:4, vol/vol), and this value was considered the initial micellar β-carotene concentration. Micelles were freshly prepared for each experiment and used within the same day.

β-carotene uptake, efflux, and transport assays. β-carotene uptake by Caco-2 cells was assayed according to a previous publication (17), with slight modifications. After two washings with 0.5 mL serum-free DMEM at 37°C, the Caco-2 monolayers in the 24-well plates were incubated for 2 h with 0.5 mL β-carotene mixed micelle preparations in serum-free DMEM. At the end of the incubation period, the plates were kept on ice while the apical medium was collected, and the monolayers were washed twice with 0.5 mL of 10 mmol/L sodium taurocholate in PBS and once more with PBS. The cells were homogenized with 1 mL PBS and 0.02 mL of 0.2 mmol/L α-tocopherol in methanol (as an antioxidant), by using a probe-type sonicator at moderate intensity. A 0.8-mL aliquot of the cell homogenate was collected, and 0.2 mL of ethanolic 0.4 mmol/L ethyl-β-apo-8′-carotenoate (internal standard) was added. Subsequently, 0.6 mL ethanol, 0.8 mL ethyl acetate, and 0.8 mL hexane were added and the mixture was vortexed after each addition. The upper phase was collected into centrifuge tubes and submitted to vacuum centrifugation. The dry residue containing β-carotene and the internal standard was then redissolved in 0.1 mL dichloromethane/methanol (1:4, vol/vol) and analyzed by HPLC. An aliquot of the apical medium was filtered through 0.2-μm PTFE membranes (Millipore, Bedford, MA). Samples of the unfiltered medium and the filtrates were subjected to β-carotene analyses by HPLC (as described above for the mixed micelles), in order to calculate the micellar (soluble) and extramicellar (aggregated, retained by the 0.2-μm membrane) β-carotene recoveries at the end of the incubation with Caco-2 cells.

For the efflux experiments, Caco-2 cells were washed twice with serum-free DMEM and incubated for 2 h with β-carotene micelles containing 50μmol/L 16:0-LysoPC and approximately 1.5μmol/L β-carotene, under the same conditions described above. After the uptake phase, the cells were washed twice with 0.5 mL serum-free DMEM, and were incubated for another 2 h (efflux period) with β-carotene-free micelles of varied phospholipid compositions. At the end of the efflux period, the media were collected and cell debris removed by centrifugation (400 × *g*, 3 min). β-carotene was extracted from a 0.3-mL aliquot of apical medium, and analyses were performed by the same procedures described above for the cell homogenates.

The uptake and transport of β-carotene across a Caco-2 monolayer was measured as previously published (19). β-carotene micelles in serum-free DMEM (1.5 mL) were added to the apical side, and 10% FBS-DMEM (2.5 mL) was added to the basolateral side. The cells were incubated for 16 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The β-carotene extraction and analyses in the cells and basolateral medium were performed as described above, adjusting the internal standard concentration and sample dilution factors as required.

HPLC analyses. The β-carotene concentration in the medium and cells was determined by HPLC (LC-10AD pump, SPD-M10A photodiode array detector, CTO-10AS column oven, Shimadzu, Kyoto, Japan). Separations were done in an ODS column (Inertsil ODS-3, 2.1 mm i.d. × 100 mm, GL Science, Tokyo, Japan), and the mobile phase consisted of methanol/ethyl acetate (60:40, vol/vol), containing 0.1% ammonium acetate, flowing at 0.2 mL/min. The β-carotene concentration was calculated from the peak area at 450 nm against a β-carotene standard curve dissolved in dichloromethane/methanol (1:4, vol/vol).

Physical characteristics of micelles. Mixed micelles were prepared as described above, but using PBS as the dissolving medium. The UV-visible spectra (250–600 nm) of β-carotene in mixed micelles were taken in a spectrophotometer (Model U-3310, Hitachi, Japan) equipped with a water-thermostatic cell holder connected to a water bath at 37°C. The spectral bandwidths were measured at the height equivalent to one-half of the absorbance at the absorption maximum (λ_{max}) of each spectrum (full width at half maximum, FWHM). For measurement of the micelle size, β-carotene-free micelles were prepared in PBS with the same lipid composition as the original micelles. Micelle size was measured with a dynamic light scattering spectrophotometer (Otsuka Electronics, Osaka, Japan) at room temperature, using a He-Ne laser source.

Statistical analyses. Data from the uptake, transport, and efflux experiments are the means ± SD of a minimum of four wells per treatment within a single experiment, which were selected from at least three independent experiments with similar results. Statistical treatments of data consisted of one-way ANOVA, followed by *post hoc* comparisons by the Tukey-Krammer method at a 95% confidence interval.

RESULTS

In order to assess the involvement of LysoPC and PC acyl moieties on β-carotene uptake, we compared the β-carotene uptake by Caco-2 cells incubated for 2 h with micelles containing 50 μmol/L of LysoPC or PC with a wide range of acyl chain lengths. Phospholipid-free (NoPL) micelles were used as controls in each experiment. Although the initial micellar β-carotene concentration was kept between 0.9 and 1.1μmol/L, the uptake varied between experiments, depending on the Caco-2 cell passage number and other factors beyond our knowledge. Therefore, statistical comparisons were always made within one experiment, with simultaneously plated cells, from the same passage number.

The present study showed that short-chain PC, namely 4:0,4:0-PC and 6:0,6:0-PC, did not reduce β-carotene uptake by Caco-2 cells (Fig. 1A). Medium-chain PC with 8:0,8:0- to 12:0,12:0- acyl chains increased β-carotene uptake, with the highest uptake rates from micelles containing 10:0,10:0-PC, followed by those containing 12:0,12:0-PC and 8:0,8:0-PC (Fig.1A). Long-chain PC inhibited β-carotene uptake by Caco-2 cells, and the lowest uptake rates were from micelles containing 16:0,18:1-PC and 16:0,18:2-PC (Fig. 1B).

Among the LysoPC class, 6:0-LysoPC, 8:0-LysoPC (data not shown), and 10:0-LysoPC (Fig. 2) did not affect β-carotene uptake from micelles, as compared with the uptake from phospholipid-free (NoPL) control micelles. With the increase of the LysoPC acyl chain length from 12:0 to 16:0, we observed a marked increase in β-carotene uptake. The β-carotene uptake from micelles containing 18:1-LysoPC, the only LysoPC within this study having an unsaturated acyl chain, was slightly lower than that from 16:0-LysoPC micelles; however, it was still significantly higher than the β-carotene uptake from NoPL micelles (Fig. 2).

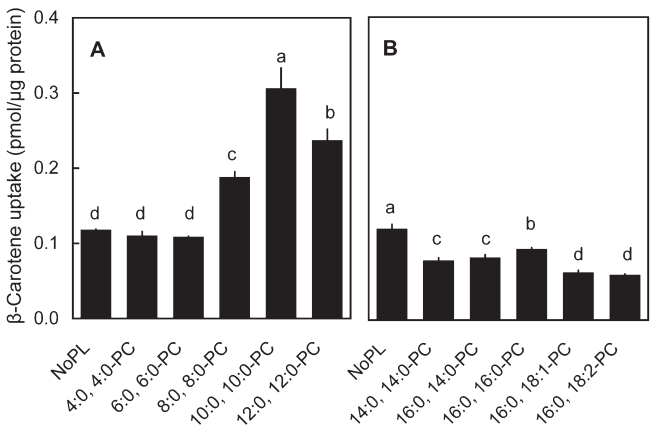

FIG. 1. Effects of short-to-medium-chain PC (A) or long-chain PC (B) on β-carotene uptake by Caco-2 cells. β-carotene (*ca.* 1 μmol/L) mixed micelles, prepared in serum-free DMEM, contained no phospholipid (NoPL) or 50 μmol/L of the corresponding phospholipid, and were incubated for 2 h at 37°C with Caco-2 cells (20–22 d post-seeding) on conventional 24-well plates. Bars represent the means ± SD of ≥ 4 wells, from a single experiment, selected from three independent experiments with similar results. Values within one graph not sharing a common letter are significantly different by the Tukey-Krammer test ($P < 0.05$).

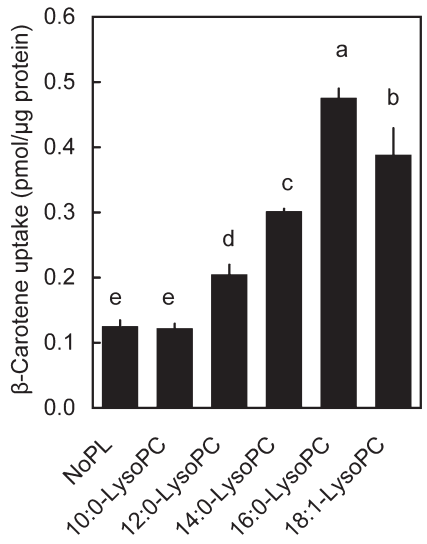

FIG. 2. Effects of medium-to-long-chain LysoPC on β-carotene uptake by Caco-2 cells. β-carotene (*ca.* 1 μmol/L) mixed micelles, prepared in serum-free DMEM, contained no phospholipid (NoPL) or 50 μmol/L of the corresponding phospholipid, and were incubated for 2 h at 37°C with Caco-2 cells (20–22 d post-seeding) on conventional 24-well plates. Bars represent the means ± SD of ≥ 4 wells, from a single experiment, selected from three independent experiments with similar results. Values not sharing a common letter are significantly different by the Tukey-Krammer test ($P < 0.05$).

TABLE 1
Effect of Phosphatidylcholine and Lysophosphatidylcholine with Various Acyl Moieties on the Micellar and Extramicellar β-Carotene Recovery in the Medium after Incubation with the Caco-2 Cells

Phospholipid type	Micellar β-carotene	Extramicellar β-carotene
Medium-to-long-chain LysoPC		
NoPL	16.0 ± 1.6^{e}	49.6 ± 1.6^{e}
10:0-lysoPC	16.4 ± 1.9^{e}	47.3 ± 1.9^{e}
12:0-lysoPC	25.8 ± 1.6^{d}	49.4 ± 1.6^{d}
14:0-lysoPC	41.8 ± 1.5^{c}	19.9 ± 1.5^{c}
16:0-lysoPC	48.4 ± 0.9^{b}	1.6 ± 0.9^{b}
18:1-lysoPC	56.2 ± 3.4^{a}	2.5 ± 3.4^{a}
Short-to-medium-chain PC		
NoPL	15.4 ± 3.1^{c}	51.0 ± 3.1^{c}
4:0,4:0-PC	17.8 ± 1.2^{c}	7.6 ± 1.2^{c}
6:0,6:0-PC	17.5 ± 1.9^{c}	9.0 ± 1.9^{c}
8:0,8:0-PC	50.9 ± 1.4^{b}	21.6 ± 1.4^{b}
10:0,10:0-PC	60.0 ± 0.9^{a}	6.1 ± 0.9^{a}
12:0,12:0-PC	63.5 ± 1.9^{a}	4.4 ± 1.9^{a}
Medium-to-long-chain PC		
NoPL	16.7 ± 2.0^{d}	52.7 ± 2.0^{d}
14:0,14:0-PC	76.2 ± 1.2^{bc}	50.7 ± 1.2^{bc}
16:0,14:0-PC	73.6 ± 1.2^{c}	39.3 ± 1.2^{c}
16:0,16:0-PC	60.3 ± 1.9^{c}	26.0 ± 1.9^{c}
16:0,18:1-PC	79.6 ± 1.0^{b}	2.2 ± 1.0^{b}
16:0,18:2-PC	84.0 ± 3.0^{a}	0.9 ± 3.0^{a}

Values are percent of the initial micellar β-carotene, reported as means ± SD of ≥ 4 wells from a single experiment, selected from three independent experiments with similar results. Values within one experiment not sharing a superscript letter are significantly different according to the Tukey-Krammer test ($P < 0.05$).

The micellar and extramicellar β-carotene recoveries in the medium at the end of the incubation with Caco-2 cells were largely influenced by the phospholipids. The 12:0- to 18:1-LysoPC significantly improved micellar β-carotene and reduced extramicellar β-carotene recoveries, in comparison with those of the NoPL micelles (Table 1). The micellar recoveries increased with the lengthening of the LysoPC acyl chains (Table 1). Short-chain PC (namely 4:0,4:0-PC and 6:0,6:0-PC) did not change micellar β-carotene recoveries, whereas medium- and long-chain PC drastically increased these values, as shown by lower extramicellar β-carotene recoveries in the medium, in comparison with the values for the NoPL micelles (Table 1). When incubated in the absence of cells, more than 95% of the initial β-carotene was retained in the micellar phase, regardless of the micelle phospholipid composition (data not shown).

The NoPL micelles had a diameter of 61.6 ± 8.9 nm, and the 16:0-LysoPC micelles were of a similar size (diameter 63.4 ± 17.7 nm) regardless of their marked improvement of β-carotene uptake. The micelles containing 10:0,10:0-PC (diameter 38.3 ± 4.9 nm) were smaller than the NoPL micelles and had higher β-carotene uptake ratios compared with those from NoPL micelles. However, the 16:0,18:1-PC micelles had the smallest diameter (15.4 ± 5.2 nm), despite the low β-carotene uptake rates from these micelles. Thus, micelle size was not associated with β-carotene uptake.

The UV-visible absorbance spectrum of β-carotene is known to change according to its interaction with the solvent or the microenvironment in which it is dissolved (21,22). Therefore, we obtained the UV-visible absorbance spectra of β-carotene in solvents and mixed micelles prepared in PBS. Compared with the typical β-carotene spectrum in hexane with λ_{max} = 449.4 nm (Fig. 3), the absorbance spectrum of β-carotene in oleic acid showed a bathochromic shift to λ_{max} = 460.0 nm. The absorption maxima of β-carotene in all micelles were similar to that observed in oleic acid. However, a broadening was observed in the spectra of the NoPL, 16:0-LysoPC, and 10:0,10:0-PC micelles, whose bandwidths (FWHM) were 94.0, 92.5, and 93.3 nm, compared with 80.0 nm of the β-carotene spectrum in oleic acid. Micelles containing 16:0,18:1-PC had a further broadened spectrum, with FWHM = 104.0 nm (Fig. 3). In addition to the increase of spectral bandwidth, 16:0,18:1-PC caused a 21.2% hypochromic effect (relative to the absorbance in oleic acid). The NoPL, 16:0-LPC, and 10:0,10:0-PC micelles exhibited 6.3–7.7% hypochromic effects.

Considering that the β-carotene uptake would result from a net positive flux into cells, a large efflux from β-carotene-loaded cells would denote a higher partitioning ratio or affinity of β-carotene toward the micelles and away from the cell membrane. The affinity between β-carotene and the micelles containing no phospholipid, 16:0-LysoPC, or 16:0,18:1-PC was evaluated by measuring the β-carotene efflux from Caco-2 cells to the micelles in the apical medium. Little efflux occurred from Caco-2 cells to the NoPL micelles (Fig. 4), whereas the 16:0-LysoPC micelles caused a larger β-carotene

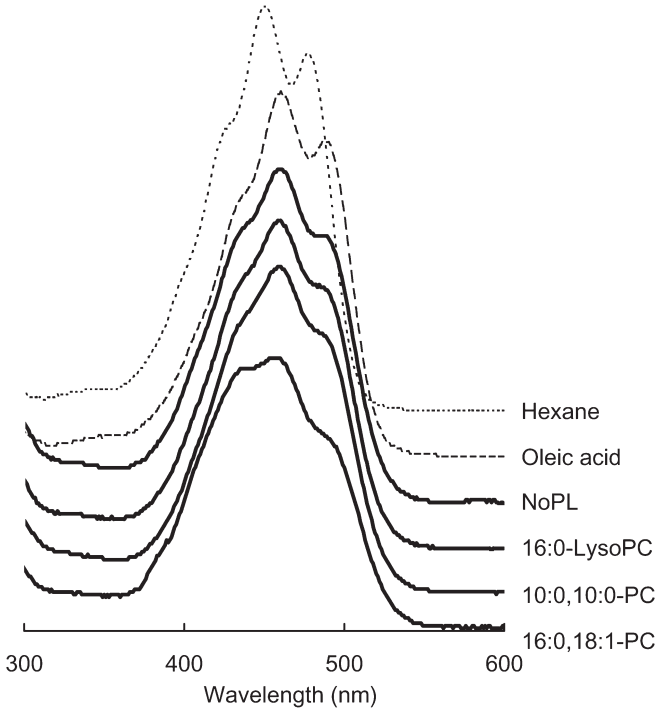

FIG. 3. UV-visible spectra of β-carotene at 37°C in hexane (dotted line; λ_{max} = 449.4 nm), oleic acid (dashed line; λ_{max} = 460.0 nm), or mixed micelles (solid lines), which were either phospholipid-free (NoPL; λ_{max} =459.5 nm) or contained 16:0-LysoPC (λ_{max} = 458.5 nm), 10:0,10:0-PC (λ_{max} = 457.3 nm), or 16:0,18:1-PC (λ_{max} = 458.7 nm). The micelles were prepared in PBS, with a lipid composition identical to that used for the uptake experiments (see Experimental Procedures). The β-carotene concentrations of all preparations were within 1.07–1.12 μmol/L.

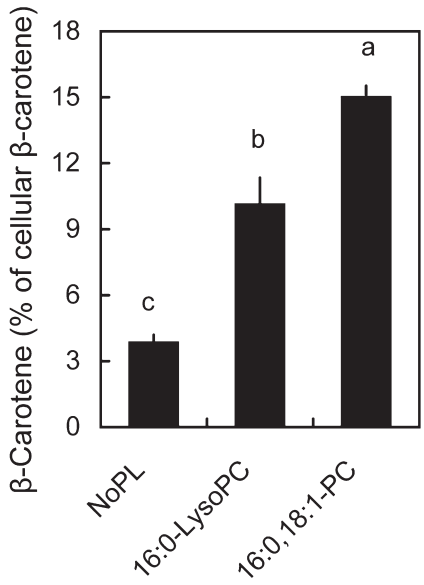

FIG. 4. β-carotene efflux from Caco-2 cells to micelles in medium containing 16:0-LysoPC, 16:0,18:1-PC, or no phospholipid (NoPL). Caco-2 cells (20–22 d post-seeding) in conventional 24-well plates were incubated for 2 h at 37°C with mixed micelles containing 1.5 μmol/L β-carotene and 50 μmol/L 16:0-LysoPC, in serum-free DMEM. After the uptake period, the cells were washed with serum-free DMEM and received β-carotene-free micelles containing none or 50 μmol/L of the corresponding phospholipid, and were incubated for 2 h at 37ºC. The β-carotene effluxed to the medium in each well was calculated as a percentage of the initial level of β-carotene accumulated in the Caco-2 cells. Bars represent the means ± SD of 4 wells, from a single experiment, selected from three independent experiments with similar results. Values not sharing a common letter are significantly different by the Tukey-Krammer test ($P < 0.05$).

efflux. The largest β-carotene efflux was observed for the 16:0,18:1-PC micelles (Fig. 4), indicating a great affinity between β-carotene and the micelles containing PC.

The β-carotene uptake during the 16-h incubation with Caco-2 cells grown in permeable supports (Transwell) showed a similar trend to that observed for Caco-2 monolayers in conventional 24-well plates (2-h incubation): 16:0,18:1-PC inhibited β-carotene uptake (although not significantly in this experiment), whereas 10:0,10:0-PC and 16:0-LysoPC enhanced it (Fig. 5). The amount of β-carotene secreted to the basolateral side was the lowest for cells incubated with NoPL micelles. The presence of 10:0,10:0-PC, 16:0,18:0-PC, or 16:0-LysoPC in the apical side significantly enhanced β-carotene secretion to the basolateral medium, with the highest values observed for cells incubated with micelles containing 16:0-LysoPC (Fig. 5). However, the amount of β-carotene secreted to the basolateral side was not proportional to its cellular uptake. The basolateral/cellular β-carotene ratio was the lowest for NoPL micelles (0.078 ± 0.012), increased significantly when 16:0-LysoPC (0.144 ± 0.010) or 10:0,10:0-PC (0.130 ± 0.020) was added to the mixed micelles, and reached the highest values when the micelles contained 16:0,18:1-PC (0.313 ± 0.026, Fig. 5), despite the low β-carotene uptake from these micelles. After the incubation with the micelles, the TEER values and the amount of triglycerides in the basolateral medium were similar in all treatments (data not shown).

DISCUSSION

Phospholipids are known to modulate the absorption of lipophilic compounds, including carotenoids. Previous studies showed that PC caused a marked inhibition of carotenoid absorption by Caco-2 cells, rats, and mice, whereas LysoPC enhanced it (12,17,18). However, the mechanisms underlying this phenomenon remain largely unknown. We hypothesized that once carotenoids are solubilized in mixed micelles, their interactions with the hydrophobic parts of micelle lipids as well as the interaction of micellar components with cell membranes may modulate the intestinal uptake of carotenoids. Therefore, we investigated the effects of micellar phospholipids and lysophospholipids with various fatty acyl moieties on β-carotene uptake by Caco-2 cells, relating the β-carotene availability to its surrounding microenvironment inside the micelles.

Despite the general knowledge that egg PC, soy PC, and pure dipalmitoyl- or palmitoyl-oleoyl-PC inhibit carotenoid uptake (13,16,23,24), and that LysoPC enhances it (12,17,18),

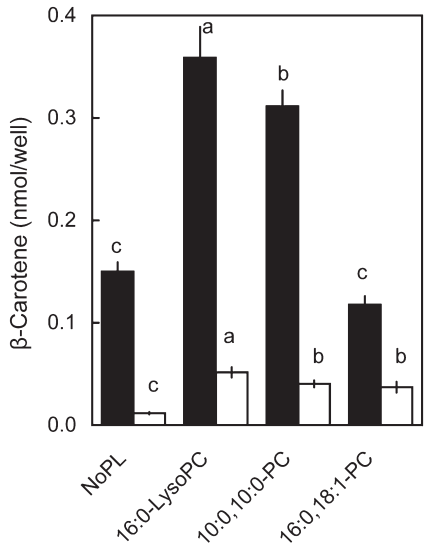

FIG. 5. Effects of 16:0-LysoPC, 10:0,10:0-PC, and 16:0,18:1-PC on β-carotene uptake and secretion to the basolateral side of Caco-2 monolayers. The mixed micelles in serum-free DMEM (1.5 mL) containing *ca.* 1 μmol/L β-carotene without phospholipid (NoPL) or with 50 μmol/L of the corresponding phospholipid were placed in the apical side of Caco-2 monolayers in 6-well Transwell plates. The basolateral side received 2.5 mL of 10% FBS-DMEM, and the cells were incubated for 16 h at 37°C. The solid bars represent the amount of β-carotene in the cells, and the open bars represent the β-carotene secreted to the basolateral medium (nmol/well). Values are means ± SD of 3–4 wells, from a single experiment, selected from three independent experiments with similar results. Values represented by the same symbols that do not share a common letter are significantly different by the Tukey-Krammer test ($P < 0.05$).

almost no information is available on the role of phospholipids' acyl groups in this event. Our results showed that in fact only medium-to-long-chain LysoPC (12:0- to 18:1-LysoPC) enhanced β-carotene uptake. Likewise, the effect of PC on β-carotene uptake was related to their acyl chain lengths. Long-chain PC (with 14:0 or longer fatty acyl chains) inhibited β-carotene uptake, whereas medium-chain PC (with 8:0 to 12:0 fatty acyl chains) enhanced β-carotene uptake by Caco-2 cells. The acyl moieties of naturally occurring PC are mostly long-chain; therefore, our results are in agreement with previous reports on the reduced uptake of lipophilic compounds by egg yolk or soy PC (23,24) However, the enhancement of β-carotene uptake by 8:0,8:0-, 10:0,10:0-, and 12:0,12:0-PC has not been previously reported. The 10:0,10:0-PC enhanced β-carotene uptake in comparable efficiency to 16:0-LysoPC. In accordance with our findings, 10:0;10:0-PC and 16:0-LysoPC were found to be equivalent enhancers of propanolol permeation across porcine buccal mucosa *ex vivo* (25) and had similar CMC, which were 5 ± 2 μmol/L for 10:0,10:0-PC (26) and 4.00 ± 0.35 μmol/L for 16:0-LysoPC (27). The CMC values decrease exponentially with the increase of methylene groups in the acyl moieties of PC and LysoPC (27), confirming that the hydrophobicity of 10:0,10:0-PC was nearly equivalent to that of 16:0-LysoPC, and providing evidence that the enhancement of the β-carotene uptake by long-chain LysoPC and medium-chain PC is related to their amphiphilic properties. Nevertheless, we cannot disregard the possibility that LysoPC may enhance β-carotene uptake by stimulating the intracellular processing of lipids, facilitating the formation and secretion of chylomicrons (16).

Medium-chain PC and long-chain LysoPC may facilitate the permeation of β-carotene by altering cell membrane properties. Short- and medium-chain PC have been reported to be very effective biosurfactants (28,29), like the long-chain LysoPC (30). 10:0,10:0-PC enhances drug absorption by the nasal epithelium (31,32), probably by altering the permeability of the cell membrane. At concentrations in the millimolar range (10^3-fold the concentrations used in our experiments), 7:0,7:0-PC solubilizes cell membranes, forming micellar aggregates (28,29), promoting cell lysis mainly by interaction with the membrane lipids. At lower concentrations, when 7:0,7:0-PC penetrates into the outer leaflet of the cell membrane, it generates a wedge-like effect due to its large polar head and short acyl chains (29). In a similar way, long-chain LysoPC would disturb membrane bilayer packing due to their cone-like shape (30). Therefore, the similar increases of β-carotene uptake by 8:0,8:0- to 12:0,12:0-PC and 14:0- to 18:1-LysoPC in our study is in agreement with the similar effects of short-to-medium-chain PC and long-chain LysoPC on membrane permeability.

Changes in the microenvironment surrounding the β-carotene molecule within the micelle structure may modulate carotenoid desorption and, therefore, cellular uptake. The UV-visible absorbance spectra of carotenoids are known to change according to the interactions between the carotenoid and its surrounding environment such as solvents (21), the interior of lipoprotein particles (33), and aggregated carotenoid molecules(34,35). The λ_{max} is proportional to solvent polarizability, and thus to its refractive index (21). The λ_{max} in the UV-visible spectra of β-carotene solubilized in mixed micelles was shifted to approximately 460 nm. A similar λ_{max} was observed when β-carotene was dissolved in oleic acid. Therefore, in our experiments β-carotene was most likely to be solubilized in a microenvironment with similar polarizability to that of oleic acid. Such environment corresponds to the micelle core, where the β-carotene molecule is surrounded by the hydrophobic moieties of oleic acid and monoolein. Further comparisons between the β-carotene UV-visible spectra in mixed micelles and those in oleic acid indicated slight higher FWHM values for the spectra of NoPL, 16:0-LPC, and 10:0,10:0-PC micelles. An additional spectral broadening and a partial loss of the vibrational fine structure were detected in the spectrum of 16:0,18:1-PC. Hashimoto *et al.* (34) reported increases in the spectral bandwidth along with increases of β-carotene aggregation levels. Therefore, in the present study, part of the β-carotene molecules may have aggregated in the micelle interior, particularly when it contained 16:0,18:1-PC, delaying β-carotene desorption from these micelles. The strong hypochromic effect caused by 16:0,18:1-PC also indicates a very distinct microenvironment surrounding the β-

carotene molecules in micelles containing this phospholipid (33).

Considering that β-carotene uptake would depend on a concentration gradient between the micellar phase in the medium and the cells, we cannot rule out the possibility that an increased micellar β-carotene recovery at the end of the incubation would favor higher uptake rates. However, the incorporation of long-chain PC (14:0,14:0-, 16:0,14:0-, 16:0,16:0-, 16:0,18;1-, and 16:0,18:12-PC) markedly enhanced micellar β-carotene recovery, without proportional increases in β-carotene uptake. This indicates that the β-carotene level in the micellar phase was not the major factor modulating the uptake. Instead, the micelle's capacity to hold or retain the carotenoid molecule and to delay its desorption to the intermicellar phase may modulate carotenoid uptake.

Smaller micelles are believed to travel faster through the unstirred water layer in the small intestine and reach the epithelial cells. Nevertheless, our data indicate that micelle size was not correlated with the β-carotene uptake by Caco-2 cells. Using segments of rabbit small intestine, Rampone and Machida (13) also found no relation between micelle size and the uptake of cholesterol.

Given that micelle size and micellar β-carotene recovery could not fully explain the effects of phospholipids on β-carotene uptake, we evaluated the affinity between β-carotene and mixed micelles by measuring β-carotene efflux from β-carotene-enriched cells back to carotene-free micelles in the apical medium. In comparison with the β-carotene efflux to NoPL and l6:0-LysoPC micelles, the larger β-carotene efflux to micelles containing 16:0,18:1-PC indicated a strong affinity between β-carotene and these micelles, shifting the partition of β-carotene from the cells toward the micelles, and supporting the hypothesis of a restricted desorption of β-carotene from mixed micelles containing long-chain PC. In agreement with these results, Hamada *et al.* (24) reported that the presence of egg yolk PC in the micelle reduced cholesterol transfer to triolein *in vitro*, and inhibited cholesterol absorption by rats and by Caco-2 cells.

Considering the lipophilic nature of carotenoids, their intracellular transport may resemble that of other lipids. However, most of the mechanisms regarding the intracellular transport of carotenoids, as well as the factors regulating its efficiency, remain unknown. We compared the effects of selected PC and LysoPC on the apical uptake and basolateral secretion of β-carotene by using Caco-2 cells grown in porous membranes (Transwell). The results for uptake resembled those from Caco-2 in conventional wells. However, compared with the NoPL control, 16:0,18:0-PC stimulated β-carotene secretion to the basolateral side, despite its low uptake. Mathur *et al.* (36) reported an increased secretion of triacylglycerol-rich lipoproteins when Caco-2 cells were incubated with di18:1-PC or egg-PC. An increase of triacylglycerol and apolipoprotein B synthesis in cells and their subsequent secretion to the basolateral side would explain the relative increase of β-carotene secretion by 16:0,18:0-PC observed in our experiment. However, we did not detect any change of triacylglycerol secretion (data not shown), and we did not assess the apolipoprotein B secretion to the basolateral medium. In our experiments, the concentration of phospholipids in the apical medium was one-fifth of that used in the study by Mathur *et al.* (36), which could explain the discrepancy with our results. Therefore, the explanation for such increase of β-carotene secretion by phospholipids awaits further investigation.

The absorption of carotenoids and other lipophilic compounds involves their solubilization in mixed micelles prior to uptake by the enterocytes. In the present study, we showed that the β-carotene uptake was largely affected by the fatty acyl moieties of phosphatidylcholines and lysophosphatidylcholines in mixed micelles. Although the entire mechanism involved in the modulation of β-carotene absorption by these phospholipids remains to be clarified, this study showed that the long acyl chains of PC molecules interact with β-carotene in the micelle interior, shifting the partition toward the micellar phase and inhibiting carotenoid uptake by enterocytes. Medium-chain PC and long-chain LysoPC have similar amphiphilic properties and may enhance β-carotene uptake by increasing cell membrane permeability.

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Fatty Acid Composition of Habitual Omnivore and Vegetarian Diets

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ABSTRACT: High-fat diets are implicated in the onset of cardiovascular disease (CVD), cancer, and obesity. Large intakes of saturated and *trans* FA, together with low levels of PUFA, particularly long-chain (LC) omega-3 (n-3) PUFA, appear to have the greatest impact on the development of CVD. A high n-6:n-3 PUFA ratio is also considered a marker of elevated risk of CVD, though little accurate data on dietary intake is available. A new Australian food composition database that reports FA in foods to two decimal places was used to assess intakes of FA in four habitual dietary groups. Analysis using the database found correlations between the dietary intakes of LC n-3 PUFA and the plasma phospholipid LC n-3 PUFA concentrations of omnivore and vegetarian subjects. High meat-eaters (HME), who consumed large amounts of food generally, had significantly higher LC n-3 PUFA intakes (0.29 g/d) than moderate meat-eaters (MME) (0.14 g/d), whose intakes in turn were significantly higher than those of ovolacto-vegetarians or vegans (both 0.01 g/d). The saturated FA intake of MME subjects (typical of adult male Australians) was not different from ovolacto-vegetarian intakes, whereas n-6:n-3 intake ratios in vegetarians were significantly higher than in omnivores. Thus, accurate dietary and plasma FA analyses suggest that regular moderate consumption of meat and fish maintains a plasma FA profile possibly more conducive to good health.

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The high dietary fat intake of Western societies has been implicated as a major cause of chronic diet-related diseases such as cardiovascular disease (CVD), which is one of the leading causes of premature death in these societies. However, the absolute intake of dietary fat may be less important than the type of fat ingested (1). Blood cholesterol levels, which are linked with risk of heart disease, are strongly related to the percentage of total dietary energy intake provided by saturated fats (2). A diet high in saturated FA (SFA) and low in PUFA is an important contributor in the onset of atherosclerosis and CVD (3). Only one SFA, stearic acid (18:0), is an exception to this, as it has no effect or actually helps to lower LDL cholesterol levels, echoing the effects of monounsaturated FA (MUFA) and PUFA (4).

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Abbreviations: AA, arachidonic acid; BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; DPA, docosapentanoic acid; HME, high meat-eater; LA, linoleic acid; LNA, alpha-linolenic acid; LC, long-chain; MME, moderate meat-eater; MUFA, monounsaturated FA; ovolacto, ovolacto-vegetarian; SFA, saturated FA.

Dietary PUFA contain two biologically important and non-interchangeable groups, the omega-3 (n-3) and omega-6 (n-6) PUFA. Numerous studies have indicated the positive effects on health of PUFA from both n-3 and n-6 families. However, the long-chain (LC) forms of n-6 PUFA (those with >18 carbon atoms) give rise to several eicosanoids with deleterious effects, whereas LC n-3 PUFA produce eicosanoids with beneficial cardiovascular effects (5). Because n-3 and n-6 FA compete for the same enzymes for conversion to the different vasoactive eicosanoids, the balance of intake of these FA will largely determine the type and amounts of eicosanoids that are present in the human body (6).

The most abundant dietary n-3 PUFA is α-linolenic acid (LNA, 18:3n-3), found mainly in plant foliage and some plant oils such as flaxseed, soybean, and canola (7). It is a metabolic precursor for LC n-3 PUFA such as EPA (20:5n-3) and DHA (22:6n-3), both of which have numerous and beneficial roles in eicosanoid formation and cell membrane function. These roles of EPA and DHA have been reviewed elsewhere (8). However the conversion of LNA to EPA and DHA in humans is notoriously poor; in males for example the conversion of dietary LNA to EPA has been shown to be of the order of 3–8%, with only minute amounts proceeding through to DHA (9). Due to the competition between linoleic acid (LA, 18:2n6) and LNA for the same elongase and desaturase enzymes, the usual high LA intakes observed in Western diets further suppresses the conversion of the relatively small dietary intake of LNA to EPA and DHA (10), although there can be substantial elongation and desaturation of LNA to EPA in diets very low in LA (11). Consumption of the foods and supplements rich in the preformed LC n-3 PUFA (EPA, DHA) lead to more significant and rapid increases in tissue levels of these LC PUFA (9). These foods include oily fish, fish oils rich in EPA and DHA, and lean meats rich in EPA and docosapentanoic acid (DPA, 22:5n-3) and seal oils rich in EPA, DPA, and DHA (12).

The importance of dietary n-3 PUFA was first highlighted by studies on Greenland Eskimos, whose consumption of large amounts of marine animals contributed to a high n-3 PUFA intake that appeared to be responsible for their low occurrence of CVD despite a high-fat diet (13). Several clinical and epidemiological studies have supported the role of n-3 PUFA in preventing CVD (14). Two of particular significance were the DART (Diet and Reinfarcation Trial), which showed a 62% reduction in CVD-related deaths in subjects consuming 450 mg/d of EPA and DHA (15) and the GISSI prevention trial, which

showed that post–myocardial infarct patients taking 850 mg/d of n-3 PUFA had a 30% reduction in coronary death over 3.5 y (16). In more recent times, LC n-3 PUFA have been shown to have some benefits in the area of psychiatric disorders (17,18).

Clinical trials have also indicated a reduction of plasma triglycerides in subjects who consumed n-3 PUFA (19), whereas consumption of LC n-3 PUFA also prolongs bleeding time by decreasing platelet activity (20) and also shows improvements in insulin sensitivity (21). However, because plants cannot convert LNA to LC n-3 PUFA, humans on plant-based diets must rely on endogenous synthesis to obtain these valuable LC n-3 PUFA (22).

The intake as well as type of n-3 PUFA recommended in different countries varies greatly, depending on the scientific rationale on which it was based (i.e., whether an intake level was selected to avoid deficiency, affect biomarkers, or prevent disease) (23). The recommended daily British intake for LC n-3 PUFA is 1,430 mg of EPA and DHA combined for men and 1,145 mg for women (23,24). An expert committee in the Netherlands recommends a daily intake of 200 mg LC n-3 PUFA (25). However, an expert committee from the International Society for the Study of Fatty Acids and Lipids (ISSFAL) promotes a minimum intake of double this amount, 500 mg/d combined EPA and DHA (26). A recent French survey found a mean intake of LC n-3 PUFA of 497 mg/d in men and 400 mg/d in women (27). These intakes essentially matched the French recommendations for LC n-3 PUFA consumption: 500 mg/d, including DHA intake of 120 mg/d, for men; and 400 mg/d, with 100 mg/d DHA intake, for women (27). The American Heart Association recommends that all adults without CHD consume approximately 1,000 mg/d of EPA and DHA combined (28). The intake for Americans is apparently below these levels, with an average total consumption of 1,600 mg/d total n-3 PUFA, mainly from LNA, with an estimate of 200 mg/d from EPA and DHA (29). Many organizations now present recommendations in terms of ratios between LA and LNA, as the major sources of n-6 and n-3 PUFA, or as a percentage of total energy intake, such as a recommendation for n-3 PUFA intake of 1–2% of daily energy for the general adult population by the World Health Organisation (30).

In Australia, the majority of n-3 PUFA in the diet is also LNA from plant matter (31). There is evidence that consumption of Australian red meat increases LC n-3 PUFA levels in plasma phospholipids (32), but there has been an overall drop in the consumption of meat as well as poultry and fish, previously the major sources of LC PUFA**,** in the Australian diet (33). The increased intake of sugar, alcohol, dairy products, and grain-based products (rich in LA), which now provide over 60% of energy in the average western diet (34), is a trend that may have negative consequences for health by reducing n-3 PUFA intake and overemphasising n-6 PUFA intake, leading to a systemic imbalance in these two FA families.

Since the 1960s, the introduction of new foods containing PUFA, such as polyunsaturated margarines and cooking oils, initially led to changes in the types of fats eaten by consumers in Australia without changing their total fat intake (35). However, a survey of dietary habits in Australia in 1993 found that fat consumption was now decreasing in the average diet, mainly because of a reduced consumption of PUFA (36). This conclusion was supported by the large National Nutrition Survey of 1995 (NNS 1995), which indicated that the total intake of PUFA in the Australian diet had decreased to 5% of total energy intake in both men and women (37), but little is known about LC PUFA intake.

The modest levels of LC PUFA present in foods have previously been difficult to measure to more than a tenth of a gram per 100 g, with lesser amounts than this recorded as zero in food composition tables and therefore discounted entirely in dietary analyses. In NUTTAB 95, a comprehensive food composition database widely used for dietary analyses in Australia (38), the previous FA supplement was limited, if not inaccurate, due to old laboratory techniques (packed-column GC) and because it related to foods present in the food supply prior to 1992 rather than current foods (39). Development of new technology now enables more accurate FA measurement and hence recording of trace LC PUFA amounts in foods. In the wake of this, the FA database recently developed at RMIT University Melbourne reports FA values in foods to two decimal places, enabling far greater accuracy in the calculation of dietary intakes of LC PUFA in individual subjects (40).

The aim of this study was to determine the amount, type, and source of FA, particularly LC PUFA, in four habitual diets consumed by healthy male Australian subjects, and to compare the dietary intakes for each individual with their plasma FA levels of corresponding FA.

EXPERIMENTAL PROCEDURES

Background. This study formed part of a larger study examining health aspects of four habitual diet groups (41). The study was approved by the Human Ethics Committee of RMIT University, and all subjects consented in writing to participation.

Subjects. Data was collected from 147 healthy male nonsmokers aged 20–55 y and originally recruited through university advertisements and local Melbourne newspapers. Selection criteria involved exclusion of volunteers if they showed evidence of CVD or had a family history of CVD, hypertension, renal disease, hyperlipemia, hematological disorders, diabetes, or excessive alcohol intake, or if they took medications or nutrient supplements (41). Eight recruited subjects were subsequently eliminated (prior to data analysis or diet group identification) due to abnormally high blood pressure and/or lipoprotein lipids or the use of medications. A final cohort of 139 healthy male subjects was investigated.

Data collection and grouping of subjects. Anthropometric data such as age, weight, height, and body mass index (BMI) were recorded for each subject and a semiquantitative food frequency questionnaire completed with assistance from trained nutritionists and a dietician. The dietary information obtained was first analyzed (41) using Diet 1 version 4 software (Xyris, Brisbane) combined with the NUTTAB 95 database, which is based on the published Composition of Foods, Australia (42).

Data was obtained for the intake of nutritional factors including total energy, total fat, and the three fat subgroups: saturated fats, monounsaturated fats, and polyunsaturated fats.

Subjects were divided into groups on the basis of their habitual food intake during the 6 mon prior to the study. When the daily meat intake of the meat-eaters was plotted in a scatter diagram, it was found that subjects divided naturally into two distinct groups: those who consumed less than 260 g/d meat (which included lean meat cuts and the meat portion of meat products, dishes, and takeaway foods) and those who consumed 285 g or more meat per day (163.2 ± 48.4 g vs. 387.2 ± 122.0 g, mean ± SD, $P < 0.001$). The two groups were therefore known as the MME and HME, although these descriptions did not take into account the relative quantities of other foods consumed. In general, the HME consumed considerably more total food by weight and energy intake on a daily basis than the MME (16.4 ± 3.3 MJ vs. 11.3 ± 2.5 MJ, $P < 0.001$). The non-meat-eaters were divided into the ovolacto-vegetarian (ovolacto) group, where subjects ate meat no more than six times per year but consumed eggs and dairy products, and the vegan group, who consumed no meat or eggs, and dairy products no more than six times per year (41). Hence, the four subject groups consisted of HME (n = 18), MME (n = 60), ovolacto (n = 43), and vegans (n = 18). However, 13 of these subjects were excluded from the dietary analysis part of the study because of incomplete or questionable dietary data (underreporting, energy intake < 95% of BMR; overreporting > 160% BMR). Hence reliable dietary intake data was collected for 126 subjects from the four subject groups, consisting of HME (n = 16), MME (n = 53), ovolacto (n = 40), and vegans (n = 17).

FA analysis. The dietary data was analyzed using the FA database developed by the RMIT University Lipid Research Group (40) (which is included in the Food Works software package, Xyris, Brisbane). The database includes sections of the NUTTAB 95 FA supplement (39) and covers 11 SFA, 7 MUFA, 10 PUFA (6 n-6 and 4 n-3), and 3 *trans* FA. FA data (g/100 g food) is presented to two decimal places for 1,044 Australian foods (concentrating on common foods, lipid-rich foods, and any foods in the Australian diet containing LC PUFA), enabling meaningful data to be obtained for the dietary LC PUFA intake of subjects. To ensure consistency of data, the total fat content generated by the FA database was compared with the NUTTAB 95 database values for individual fat content of foods. Also the total fat consumption derived from analysis of each subjects food frequency questionnaire using the new FA database was validated against the NUTTAB 95 dietary analysis for total fat intake.

Where study subjects had consumed foods that were not listed in the FA database, these were replaced during analysis (following standard dietary analysis procedures) by a related food with a similar FA profile (e.g., Weetbix entered as "wheat breakfast biscuit"; milkshake entered as "milk, flavored, chocolate, fluid"). Replacement was not always possible, as many complex foods consumed by subjects were not present in the FA database. In that case, "recipes" were created using ingredients already present in the FA database to produce replacement food items containing similar overall total fat contents and FA profiles. The new food items were then added to the database. However, these food items were all low-fat with minimal LC PUFA content; thus, any inaccuracy involved in this process had minimal impact on the accumulated LC PUFA content of the habitual diets. The dietary intake data for all the subjects, and subsequently their FA profiles, were grouped according to their habitual diet. The mean total fat intake and intake of each FA was then calculated for each dietary group.

Plasma phospholipid FA determination. Plasma was obtained from citrated (overnight fasted) whole blood by centrifugation at 3,000 rpm for 15 min at 4°C. Plasma phospholipid (PL) fractions were separated by TLC. The FAME of the plasma phospholipids were prepared by saponification using potassium hydroxide (0.68 mol/L methanol) and transesterification with 20% boron trifluoride in methanol. The FA compositions of plasma phospholipids were determined by gas-liquid chromatography as previously described (41).

Statistical analysis. One-way ANOVA was carried out (SPSS v. 12, SPSS Inc, Chicago, IL) to compare the variability in scores for each FA intake between the four habitual diet groups. The Levene test for homogeneity of variance determined that all the FA intakes had a significantly different variance ($P < 0.05$) when a Bonferroni correction was applied, except for LNA ($P = 0.481$). The Tamhane and Dunnett T3 post-hoc tests were applied (using a significance level of $P < 0.05$) to the FA with non-equal variance. The equal-variance Tukey's HSD and Scheffe post-hoc tests were applied to the LNA data (significant at $P < 0.05$).

Regression analysis was carried out (SPSS v. 12) to determine whether there was a significant correlation between the subjects' mean intake levels (g/d) and their plasma phospholipid concentrations (mg/100 mL) of specific FA. Preliminary analyses were performed to ensure no violation of the assumption of normality, linearity, and homoscedasticity in the data. This involved generating a scatter plot for each correlation and inspecting the distribution of the data points to check for outliers, determining the direction of the relationship between the variables, confirming linearity, and ensuring that there were no unpaired data for the two variables.

RESULTS

The age, anthropometric, and blood pressure characteristics of each group (Table 1), have been published in detail previously (41). Both vegetarian groups had significantly lower BMI than the omnivore groups but there were no differences between the groups in systolic blood pressure, although diastolic pressures were lower in the ovolacto group than in the omnivore groups.

As explained in the Methods section, HME consumed significantly more meat, both white and red meat, than the MME group (Table 2). Vegans did not consume any meat, whereas the ovolacto group did not consume red meat but some individuals had occasional small intakes of white meat or fish. The HME group had a significantly higher energy intake than each

TABLE 1
Age, Anthropometric, and Blood Pressure Characteristics of Subjects in the Four Dietary Groups

Characteristic	High-meat group (HME) (n = 18)	Moderate-meat group (MME) (n = 60)	Ovolacto group (n = 43)	Vegan group (n = 18)
Age (years)	34.2 ± 9.4[a]	38.3 ± 7.3[b]	34.9 ± 9.0[a]	33.0 ± 7.7[a]
BMI	27.0 ± 3.4[a]	26.4 ± 3.4[b]	23.6 ± 2.8[c]	23.3 ± 3.5[c]
Waist/hip ratio	0.88 ± 0.06[a]	0.88 ± 0.05[a]	0.86 ± 0.04[b]	0.85 ± 0.05[b]
Systolic BP	129.8 ± 14.6	125.7 ± 10.5	124.4 ± 13.4	122.1 ± 9.7
Diastolic BP	83.4 ± 8.0[a]	81.7 ± 7.9[a]	77.7 ± 10.4[b]	77.7 ± 7.8[b]

Values are mean ± SD. Values in the same row that have different superscripts are significantly different from each other ($P < 0.01$).

of the other three dietary groups, and HME subjects consumed, as a percentage of energy intake, significantly more protein and fat and less carbohydrate than the other groups. Nonetheless, in all groups, the largest percentage of energy intake was supplied by carbohydrates, followed by fats and then proteins. The high energy intake and total fat intake of the HME was derived from their substantial consumption of all kinds of food, not just their meat intake (43).

Daily FA intakes. Table 3 displays the daily dietary intakes of specific FA for each of the four dietary groups. There was a significant difference ($P < 0.05$) between the dietary group values for myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), and total SFA. Compared with the other groups, the HME group consumed significantly more of each SFA and had the largest total intake of saturated fats (77.17 ± 33.81 g/d). The vegan group consumed significantly less of each SFA than the other groups and had the lowest total intake (16.71 ± 6.16 g/d). There was no significant difference between the individual and total SFA consumptions of the MME group (35.24 ± 15.81 g/d) and the ovolacto group (32.40 ± 14.51 g/d). The predominant SFA consumed by each dietary group was palmitic acid (16:0).

There was a significantly higher intake ($P < 0.05$) of both oleic acid (18:1) and total MUFA in the HME group compared with the other three groups (Table 3).

The intake of LA was lowest in the MME group (10.90 ± 5.69 g/d) by a significant margin ($P < 0.05$), whereas the other three groups had similar intakes (Table 3).

The HME group consumed significantly more LNA (1.96 ± 0.66 g/d) than any other group (Table 3), which appeared to be derived both from their higher intake of foods generally, including plentiful meats and some fish, and from a larger consumption of canola-based margarine and oils (43).

The intake of AA was significantly different for each of the four dietary groups, the only FA where this was the case. As AA is found only in animal tissue (44), it was not surprising that HME subjects consumed the largest amounts (0.24 ± 0.11 g/d), followed by the MME group (0.10 ± 0.04 g/d), ovolacto group (0.03 ± 0.03 g/d), and vegan group (0.00 ± 0.00 g/d) (Table 3).

Similarly, EPA and DPA, which are both present mainly in marine vertebrates (particularly deep-sea fish) and to a lesser extent terrestrial animals, were not consumed in measurable quantities by the ovolacto or vegan groups (Table 3). HME subjects consumed significantly more EPA (0.07 ± 0.02 g/d) and DPA (0.09 ± 0.05 g/d) than the MME group (0.04 ± 0.03 g/d and 0.03 ± 0.02 g/d, respectively) (Table 3).

There was no significant difference between the two omnivore groups in their intake of DHA, which is found mainly in foods of marine origin and eggs. The daily intakes of 0.12 ± 0.10 g for HME and 0.07 ± 0.10 g for MME subjects were significantly greater than that of the ovolacto subjects (0.01 ± 0.01 g), who most likely consumed DHA in the form of eggs, which contain approximately 238 mg DHA/100 g (12). Vegan subjects consumed no DHA at all (Table 3).

The total n-3 intake was significantly higher for the HME group (2.25 ± 0.74 g/d) compared with the three other groups,

TABLE 2
Selected Daily Dietary Factors of the Four Habitual Diet Groups

Dietary Factors	High-meat group (HME) (n =18)	Moderate-meat group (MME) (n = 60)	Ovolacto group (n = 43)	Vegan group (n = 18)
White meat (g)	109.6 ± 51.6[a]	53.7 ± 27.1[b]	0.2 ± 0.7[c]	0.0 ± 0.0[c]
Red meat (g)	270.4 ± 118.0[a]	108.9 ± 41.1[b]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
White and red meat (g)	387.2 ± 122.0[a]	163.2 ± 48.4[b]	0.2 ± 0.7[c]	0.0 ± 0.0[c]
Fish (g)	27.7 ± 23.9[a]	26.1 ± 18.3[a]	1.4 ± 3.4[b]	0.7 ± 1.8[b]
Energy (MJ)	1.64 ± 3.31[a]	1.13 ± 2.48[b]	1.15 ± 2.46[b]	1.18 ± 2.44[b]
Protein (% of energy)	19.4 ± 1.3[a]	17.9 ± 2.2[b]	14.7 ± 2.2[c]	14.1 ± 2.4[c]
Carbohydrate (% of energy)	40.3 ± 4.4[a]	45.7 ± 6.8[b]	50.9 ± 6.4[c]	57.4 ± 5.4[d]
Total fat (% of energy)	37.8 ± 4.2[a]	32.8 ± 6.1[b]	32.7 ± 6.2[b]	28.2 ± 4.3[c]

Values are mean ± SD. Values in the same row that have different superscripts are significantly different from each other ($P < 0.01$).

TABLE 3
The Daily Dietary Consumption of Selected FA for the Four Dietary Groups

FA	High-meat group (HME) (n = 16)	Moderate-meat group (MME) (n = 53)	Ovolacto group (n = 40)	Vegan group (n = 17)
14:0	8.33 ± 4.89[a]	3.70 ± 1.98[b]	3.21 ± 1.97[b]	0.95 ± 0.70[c]
16:0	39.57 ± 16.29[a]	18.32 ± 8.41[b]	17.34 ± 7.39[b]	10.19 ± 4.20[c]
18:0	20.22 ± 8.11[a]	9.05 ± 3.93[b]	7.21 ± 3.29[b]	3.19 ± 1.75[c]
Total SFA	77.17 ± 33.81[a]	35.24 ± 15.81[b]	32.40 ± 14.51[b]	16.71 ± 6.16[c]
18:1	58.55 ± 20.84[a]	30.63 ± 11.63[b]	34.23 ± 12.65[b]	29.17 ± 9.16[b]
Total MUFA	63.72 ± 22.47[a]	32.98 ± 12.40[b]	36.64 ± 13.33[b]	31.22 ± 10.12[b]
18:2n-6 (LA)	20.29 ± 10.39[a]	10.90 ± 5.69[b]	18.43 ± 8.61[a]	21.60 ± 9.59[a]
18:3n-3 (LN)	1.96 ± 0.66[a]	1.27 ± 0.56[b]	1.42 ± 0.70[b]	1.15 ± 0.75[b]
20:4n-6 (AA)	0.24 ± 0.11[a]	0.10 ± 0.04[b]	0.03 ± 0.03[c]	0.00 ± 0.00[d]
20:5n-3 (EPA)	0.07 ± 0.02[a]	0.04 ± 0.03[b]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
22:5n-3 (DPA)	0.09 ± 0.05[a]	0.03 ± 0.02[b]	0.00 ± 0.01[c]	0.00 ± 0.00[c]
22:6n-3 (DHA)	0.12 ± 0.10[a]	0.07 ± 0.10[a]	0.01 ± 0.01[b]	0.00 ± 0.00[c]
Total PUFA	23.00 ± 11.13[a]	12.48 ± 5.96[b]	19.90 ± 8.88[a]	22.75 ± 10.08[a]
Total n-3 PUFA	2.25 ± 0.74[a]	1.14 ± 0.61[b]	1.43 ± 0.70[b]	1.22 ± 0.75[b]
Total n-6 PUFA	20.75 ± 10.49[a]	11.07 ± 5.71[b]	18.46 ± 8.61[a]	22.87 ± 9.59[a]
Total LC n-3 PUFA	0.29 ± 0.15[a]	0.14 ± 0.14[b]	0.01 ± 0.01[c]	0.01 ± 0.00[c]
n6:n3	9.2: 1	9.7: 1	12.9: 1	18.7: 1

Values are g/d, mean ± SD. Values in the same row that have different superscripts are significantly different from each other ($P < 0.01$).

whose intakes were not significantly different (MME: 1.14 ± 0.61 g/d; ovolacto: 1.43 ± 0.70 g/d; and vegan:1.22 ± 0.75 g/d). In each group, the total n-3 intakes were mainly in the form of LNA.

Total n-6 intakes, however, were similar in the HME, ovolacto, and vegan groups (20.75 ± 10.49, 18.46 ± 8.61, and 22.87 ± 9.59 g/d, respectively). All three groups consumed significantly more n-6 FA than the MME group (11.07 ± 5.71 g/d), primarily due to the higher intake of LA in these three groups.

All groups consumed some LC n-3 PUFA. The modest intake in the ovolacto (0.01 ± 0.01 g/d) and vegan subjects (0.01 ± 0.00 g/d) was most likely due to the occasional consumption of small amounts of fish by some vegans and the contribution of DHA from eggs consumed by the ovolacto group. The daily LC n-3 PUFA intake in the HME group (0.29 ± 0.15 g/d) was greater than in the MME group (0.14 ± 0.14 g/d), although foods containing these FA, including red meat and oily fish, were consumed by both groups. The HME group simply consumed greater quantities.

Importantly, the dietary intake ratio of n-6:n-3 FA became larger as the meat intake of the groups decreased. Hence, the ratios were 9.2:1 in HME subjects, 9.7:1 in MME subjects, 12.9:1 in ovolacto subjects, and 18.7:1 in vegan subjects (Table 3).

Plasma phospholipid concentrations of selected FA. The plasma phospholipid (PL) levels of each of the FA were determined for all subjects in the study. These results have been previously reported by Li *et al.* (41), and an abbreviated version is shown in Table 4.

Plasma PL total SFA levels were slightly but significantly lower in the vegan group (40.0 ± 1.9 mg/100 mL) than in the other groups, whereas total PUFA was highest in the vegan group (44.8 ± 1.3 mg/100mL). Total MUFA levels showed no significant difference between groups.

There were higher arachidonic acid PL levels in the HME subjects (10.6 ± 1.6 mg/100 mL) and MME subjects (10.5 ± 1.7 mg/100 mL) than in the ovolacto group (9.5 ± 1.9 mg/100 mL), but not the vegan group (10.6 ± 1.5 mg/100 mL) (Table 4).

EPA PL levels for the HME group (1.1 ± 0.5 mg/100 mL) and MME group (1.0 ± 0.3 mg/100 mL) were significantly greater ($P < 0.001$) than for the ovolacto or vegan groups (0.7 ± 0.3 and 0.6 ± 0.3 mg/100 mL, respectively) (Table 4). This was also the case for DHA (HME, 3.4 ± 1.0 mg/100 mL; MME, 3.3 ± 0.8 mg/100 mL; ovolacto, 2.2 ± 0.07 mg/100 mL; vegan, 2.0 ± 0.4 mg/100 mL; $P < 0.001$ for the omnivore vs. vegetarian groups). The same pattern was observed for DPA (HME, 1.3 ± 0.2 mg/100 mL; MME, 1.2 ± 0.2 mg/100 mL; ovolacto, 1.1 ± 0.2 mg/100 mL; vegan, 1.0 ± 0.3 mg/100 mL; $P < 0.01$ for the omnivore vs. vegetarian groups). Total n-6 PUFA plasma PL levels were significantly higher in the vegetarian groups, and total n-3 PUFA levels significantly higher in the omnivore groups.

Comparison of FA intake and plasma phospholipid concentrations. The dietary intake ratios (Table 3) of n-6:n-3 PUFA increased across the groups as the quantity of animal foods in the diets decreased (HME 9.2:1, MME 9.7:1, ovolacto 12.9:1, vegan 18.7:1). This pattern was also observed in the plasma PL levels (HME 5.9:1, MME 6.4:1, ovolacto 9.4:1, vegan 10.2:1). The mathematical relationship between the FA consumed and the plasma PL concentrations of these same FA in individual subjects was assessed using regression analysis (Table 5). Several significant positive correlations were found when data from all the subjects (both the omnivore and the vegetarian dietary groups) were combined.

There were no significant correlations between FA intake and plasma PL FA level for myristic acid (14:0), palmitic acid (16:0), LA, or LNA. Although stearic acid (18:0) was the only SFA that

TABLE 4
Plasma Phospholipid FA Concentrations of the Four Dietary Groups

FA	High-meat group (n = 18)	Moderate-meat group (n = 60)	Ovolacto group (n = 43)	Vegan group (n = 18)
14:0	0.3 ± 0.01	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16:0	27.2 ± 0.9[a]	27.3 ± 1.3[a]	27.0 ± 1.4[a]	26.4 ± 1.6[b]
18:0	13.3 ± 1.1	13.1 ± 0.9	13.1 ± 1.0	13.2 ± 1.0
20:0	0.3 ± 0.2[a]	0.3 ± 0.1[a]	0.2 ± 0.1[b]	0.1 ± 0.0[c]
Total SFA	41.2 ± 1.4[a]	40.9 ± 1.2[a]	40.6 ± 1.4[a]	40.0 ± 1.1[b]
18:1n-9c	9.8 ± 1.1	9.4 ± 1.4	9.6 ± 1.2	9.6 ± 0.9
18:1n-7c	1.4 ± 0.2[a]	1.5 ± 0.2[a]	1.4 ± 0.2[a]	1.7 ± 0.5[b]
Total MUFA	12.8 ± 1.0	12.6 ± 1.2	12.6 ± 1.3	12.6 ± 1.2
18:2n-6 (LA)	20.6 ± 2.6[a]	22.4 ± 2.8[b]	26.0 ± 3.1[c]	26.1 ± 2.5[c]
18:3n-3 (LN)	0.2 ± 0.1[a]	0.2 ± 1.0[a]	0.3 ± 0.1[b]	0.3 ± 0.1[b]
20:4n-6 (AA)	10.6 ± 1.6[a]	10.5 ± 1.7[a]	9.5 ± 1.9[b]	10.6 ± 1.5[a]
20:5n-3 (EPA)	1.1 ± 0.5[a]	1.0 ± 0.3[a]	0.7 ± 0.3[b]	0.6 ± 0.3[b]
22:5n-3 (DPA)	1.3 ± 0.2[a]	1.2 ± 0.2[a]	1.1 ± 0.2[b]	1.0 ± 0.3[b]
22:6n-3 (DHA)	3.4 ± 1.0[a]	3.3 ± 0.8[a]	2.2 ± 0.7[b]	2.0 ± 0.4[b]
Total PUFA	41.5 ± 2.0[a]	42.7 ± 1.8[b]	43.8 ± 2.2[c]	44.8 ± 1.3[c]
Total n-3 PUFA	6.0 ± 1.3[a]	5.8 ± 1.0[a]	4.2 ± 1.0[b]	4.0 ± 0.7[b]
Total n-6 PUFA	35.5 ± 2.1[a]	37.0 ± 2.1[b]	39.5 ± 2.6[c]	40.8 ± 1.7[d]
Total LC n-3 PUFA	5.8	5.5	4.0	3.6
n6:n3	5.9:1	6.4:1	9.4:1	10.2:1

This data was adapted, with permission from Dr. Duo Li, from previously reported data (Li *et al.*, 1999). Values are mg/100 mL, mean ± SD Values in the same row that have different superscripts are significantly different from each other ($P < 0.01$).

showed a significant correlation for the combined groups (r = 0.312, $P < 0.01$), the intake values for total SFA were also significantly correlated with plasma PL levels (r = 0.194, $P < 0.05$) (Fig. 1). A significant association was shown between the dietary intake and plasma PL levels of oleic acid (18:1), AA, EPA, DPA, and DHA ($P < 0.01$) (Table 5). Total LC n-3 PUFA intake was also significantly correlated with plasma PL levels for the combined group (r = 0.474, $P < 0.01$) (Fig. 2).

DISCUSSION

A low intake of LC n-3 PUFA and a high n-6:n-3 intake ratio have been indicated to be markers of elevated risk of CVD (3,45). Conversely, results from experimental, epidemiological, and cross-cultural studies suggest that consumption of foods rich in PUFA, particularly the LC n-3 PUFA, has a positive effect on health and assists in the prevention of chronic disease (14,46). The aim of this study was to investigate the FA composition of habitual diets of healthy male subjects, particularly their average intakes of LC n-3 PUFA, compare the intake level with selected international dietary n-3 PUFA recommendations, and correlate intake levels with plasma PL levels.

The HME food and energy intakes were much greater than those of most Australians (16.4 MJ/d compared to an average of 11.0 MJ/d for Australian males aged 19 y and over) (37), and therefore this groups' dietary FA intakes would not be typical of Australian intakes. Of the four dietary groups investigated,

TABLE 5
Correlations Between the Dietary Intakes and Plasma Phospholipid Concentrations of Selected FA in Combined Dietary Groups

FA	All subjects (n = 126)
16:0	NS
18:0	$r = 0.312^b$
Total SFA	$r = 0.194^a$
18:1	$r = 0.188^a$
Total MUFA	NS
20:4n-6 (AA)	$r = 0.275^b$
20:5n-3 (EPA)	$r = 0.394^b$
22:5n-3 (DPA)	$r = 0.344^b$
22:6n-3 (DHA)	$r = 0.409^b$
Total PUFA	NS
Total LC n-3 PUFA	$r = 0.474^b$

The degree of significance is indicated by the following: $^aP < 0.05$, $^bP < 0.01$. NS, not significant.

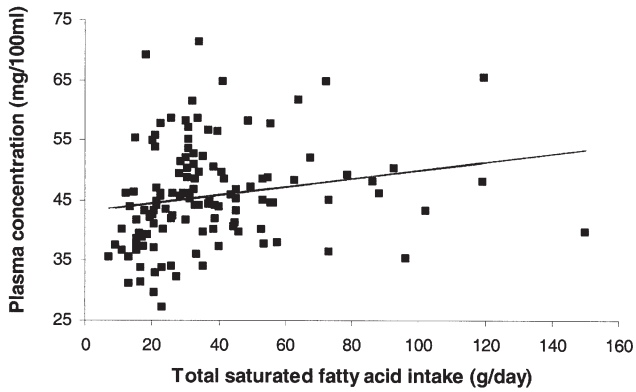

FIG. 1. Comparison of the dietary intake and plasma phospholipid concentrations of total SFA for omnivore (n = 69) and vegetarian (n = 54) subjects combined (n = 123; r = 0.194; P < 0.05).

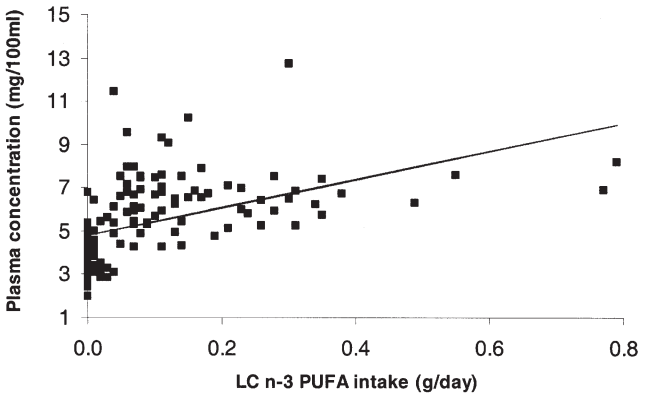

FIG. 2. Comparison of the dietary intake and plasma phospholipid concentrations of total LC n-3 PUFA for omnivore ($n = 69$) and vegetarian ($n = 54$) subjects combined ($n = 123$; $r = 0.474$; $P < 0.01$).

the food intake of the MME group (11.3 MJ/d) most closely resembled that of adult male Australians, as presented in the NNS 1995 survey (37). The mean MME group consumption of fat, 96.2 g/d and 32.8% of energy intake, was almost identical to the Australian NNS 1995 figures of 98.5 g/d fat intake and 32.4% of energy intake for adult men (aged 19 y and older) (37).

The MME group consumed significantly less LA, than the other three groups. This is explained by LA being the most abundant dietary n-6 PUFA, found largely in plant matter such as grains and seeds (47). Subjects who ate more food overall (the HME group) or more plant matter (the ovolacto and vegan groups) than the MME subjects, would indeed consume larger amounts of this FA. LNA, which is found in a variety of cereal- and vegetable-based foods as well as meats (47), was consumed in significantly higher amounts by the HME group than the other three dietary groups, due to their overall larger food intake. However, LNA was the only n-3 FA contributing substantial amounts (g/d) to the total n-3 FA intake of the two vegetarian groups, as the LC n-3 PUFA (EPA, DPA, and DHA), are found in foods of animal origin and thus were not included to any significant extent in their diet.

The AA intake was significantly different in all four groups and corresponded to the amount of meat consumed: HME subjects had the highest AA consumption whereas vegans had a zero intake (Table 3). These findings support the suggestion that diets rich in meat contribute to the high-AA tissue content of people in Westernized societies (5,12,48). Findings by Meyer *et al.* (47) indicated that Australian adults consume 52 mg of AA per day, but the two omnivore groups in this study consumed approximately two to four times this amount: 100 mg/d for MME and 240 mg/d for HME. The MME values, however, are similar to previous Australian studies, which found that adult males consumed approximately 130 mg/d of AA (12) up to 153mg/d (49).

The total n-3 PUFA (1.14 g/d) and LC n-3 PUFA (140 mg/d) intakes of the MME subjects (who would most closely resemble that of the average Australian adult) were both significantly greater than those of the vegetarian groups, although significantly less than the mean HME intakes (2.25 g/d and 290 mg/d, respectively). Two evaluations of the dietary intake data from NNS 1995 using this new Australian FA database indicated that the average Australian male was consuming 1.17 g/d of LNA and 189 mg/d of LC n-3 PUFA (47), or more recently 1.07 g/d of LNA and 246 mg/d LC n-3 PUFA (49), figures similar to those obtained in this present smaller study. These results also closely resemble figures estimated by Kris-Etherton *et al.* for intakes in the United States, of 1.6g/d of total n-3 PUFA, of which 0.1–0.2 g/d were EPA and DHA (29).

The MME total n-3 PUFA intake was equal to the lower dietary levels recommended for Canada (1.1–1.6 g/d) (50) and slightly less than the recommendation in Japan (1.6 g/d) (51). The daily LC n-3 PUFA intake of MME subjects fell below the various recommended intake levels, such as the European Academy of Nutrition recommendation of 210 mg/d (DPA, EPA plus DHA) (23), the British Nutrition Foundation level of 1–2 g EPA + DHA (24), the 500 mg/d (combined DHA and EPA) recommended by ISSFAL (26), and the 750–1,000mg/d suggested by Hibbeln for lowering the risk of psychiatric disorders (52). The recently released Nutrient Reference Values for Australia and New Zealand recommend adult male intakes of 1.3 g/d LNA and 160 mg/d LC n-3 PUFA (53). The HME group, being representative of individuals in society who consume high-energy diets, including substantial amounts of red meat, were able to meet some but not all of these recommendations for both total n-3 PUFA and LC n-3 PUFA intake. However this dietary approach is certainly not recommended by the authors. Neither vegetarian group consumed LC n-3 PUFA in amounts close to those suggested by these various authorities.

This study, even though small in size, was able to show that dietary intake of LC FA has a direct effect on systemic levels, as indicated by fasting plasma PL levels of these FA (Table 5, Fig. 2). The fact that the study consisted of differing habitual diets with very different LC PUFA intakes highlighted the impact that these intake levels can have on tissue levels of these FA. A recent study has similarly demonstrated positive correlations for LC n-3 PUFA (total LC n-3 PUFA, EPA, and DHA) but no correlations between LA or LNA intake and plasma concentrations in Japanese subjects (54).

One limitation of this study was that although subjects consumed a wide variety of different foods, only a limited number of foods (1,044 items) were available in the FA database. As a result, some items had to be substituted with another similar food, or new recipes created to resemble as closely as possible, but not necessarily duplicate, the FA profile of new foods. This may have limited the identification of true total intakes of the various FA by subjects. Secondly, this study involved an unequal number of participants in the various dietary groups (16 HME, 53 MME, 40 ovolacto, and 17 vegan subjects). Despite the limiting factors in this study, the use of the new Australian FA database, accurate to 10 mg/100 g food item (40) and a dietary data tool (FFQ) specifically designed to isolate information on lipid amount and type in the diet, means that the data reported here, particularly for LC n-3 PUFA is of a high degree of accuracy.

It was perhaps surprising to find that the total dietary intake of SFA was not significantly different in the omnivore MME

group and the ovolacto group. This was also true of the MUFA. The intake values for the individual LC PUFA, as well as total LC n-3 PUFA, were higher in the MME group than in the two vegetarian groups, as were the plasma PL levels of LC n-3 PUFA. Roshanai and Sanders (1984) also were able to show a zero intake of all LC PUFA in British vegans and intakes of LA (26.0 g/d) and LNA (1.5 g/d) similar to this more recent Australian study (21.6 g/d and 1.2 g/d, respectively). They also reported a LC n-3 PUFA intake of 510 mg/d in omnivore subjects, however it should be noted that only 10 subjects were assessed, and a small number of these consumed considerable levels of fish, thus limiting the representative nature of the population group (55). A more recent British study also reported lower proportions of plasma EPA and DHA in vegetarians and vegans compared to omnivores (56). Although the total n-3 PUFA intakes were similar in the MME group and both vegetarian groups (due to LNA levels), total n-6 PUFA intakes were significantly higher in the vegan and ovolacto groups because of their larger intakes of LA. These total values resulted in n-6:n-3 intake ratios of 9.7:1 in the MME subjects, 12.9:1 in the ovolacto subjects, and 18.7:1 in the vegan group. Because a high n-6:n-3 FA ratio is considered to be a risk factor for CVD (48), the results suggest that the consumption of red meats, which provide useful amounts of LC n-3 FA such as EPA and DPA (12), can contribute to a healthier balance of FA without necessarily supplying elevated levels of saturated fats. This may be particularly true in Australia, where beef cattle and sheep are raised predominantly on pasture and their meat contains some EPA and DPA (47) as well as very little marbling with the visible fat that provides high levels of SFA (57,58). Meat from beef raised on grain contains lower levels of n-3 FA (12). However, even small amounts of n-3 PUFA appear to be very effective in promoting an improved n-6:n-3 ratio and preventing CHD (59). The MME subjects' intake of 140 mg/d (likely to be typical for an adult Australian male), did not reach the levels recommended by many nutrition authorities. However, omnivores had significantly higher LC n3 PUFA intake than those of the ovolacto and vegan groups who consume minimal preformed LC n3 PUFA.

In conclusion, use of a new FA database of Australian foods allowed the investigation of the FA intake of subjects consuming four different habitual diets. The diet of subjects had a significant effect on their FA intake profiles and in many cases the plasma PL concentrations of those FA. Omnivore subjects consuming moderate amounts of meat (and other foods) had similar intakes of saturated and MUFA to vegetarian subjects but significantly higher intakes of total LC n-3 PUFA and a reduced n-6:n-3 intake ratio. Despite a continuing perception, even among health professionals, that consumption of red meat is deleterious to health (60), our findings do not support this argument. Rather, the data suggest that inclusion of lean red meat in the diet may actually increase the dietary intake and plasma PL ratio of n3:n6 PUFA. Consumption of lean red meat will also contribute significantly to daily intakes of protein, vitamin B12, niacin, iron, and zinc (57). Further, when consumed as part of a diet low in saturated fat, lean red meat does not increase plasma cholesterol levels or thrombotic risk factors (8). Public health messages to consume more lean meats and fish in combination with other nutritious foods could thus result in improved health outcomes in the community.

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The Hypertriglyceridemic Waist Phenotype Is a Predictor of Elevated Levels of Small, Dense LDL Cholesterol

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ABSTRACT: The hypertriglyceridemic waist (HTGW) phenotype (hypertriglyceridemia and increased waist circumference) has been proposed as an inexpensive tool to monitor individuals with the atherogenic metabolic triad, hyperinsulinemia, hyperapobetalipoproteinemia, and increased levels of small, dense LDL (sdLDL) particles. We assessed the association of the HTGW phenotype with the metabolic syndrome (MetSyn) and the atherogenic metabolic triad in inhabitants (n = 260) of northwestern Greece attending the Outpatient Lipid Clinic of the University Hospital of Ioannina. The LDL subfractions were assessed using the Lipoprint LDL System. HTGW (+) individuals had a more adverse lipid and lipoprotein profile compared with HTGW (–) individuals. Moreover, HTGW (+) subjects had elevated levels of sdLDL-C, as well as decreased mean and peak LDL particle size compared with HTGW (–) subjects. To our knowledge, this is the first report documenting the sdLDL-C abnormality in HTGW (+) subjects. Among men (n = 105), 52.3% of the MetSyn (+) individuals and 66.7% of the HTGW (+) individuals had the metabolic triad. Among women (n = 155), the corresponding percentages were 42.3% and 50.0%. Only 22.2% and 10.6% of the MetSyn (–) subjects (men and women, respectively) and 19.6% and 15.2% of the HTGW (–) subjects (men and women, respectively) had the atherogenic metabolic triad. In conclusion, the HTGW (+) phenotype is associated with a hostile lipid profile that includes higher levels of sdLDL-C and decreased LDL particle size. The HTGW phenotype, compared with the MetSyn criteria, can provide an easy and inexpensive tool to monitor patients characterized by an adverse lipid and lipoprotein profile.

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The hypertriglyceridemic waist (HTGW) phenotype has been proposed as an inexpensive tool to monitor individuals characterized by the atherogenic metabolic triad, hyperinsulinemia, elevated apolipoprotein B levels, and increased concentrations of small, dense LDL (sdLDL) particles, in men (1). Similarly, the HTGW phenotype was associated with the atherogenic metabolic triad as well as with coronary heart disease (CHD) risk factors in apparently healthy women (2). Furthermore, the results of the Hoorn study indicated that men and women with the HTGW phenotype were at increased risk for cardiovascular disease (CVD), even if they had normal glucose metabolism (3). Tanko *et al.* also reported that postmenopausal women with enlarged waist and elevated triglycerides (TG) were at increased risk for fatal CVD events; this risk was even higher than that observed in women with the metabolic syndrome (MetSyn) (4).

MetSyn represents a constellation of lipid and nonlipid CVD risk factors, and it has been recognized by the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III as a secondary target for hypolipidemic therapy (5,6). Furthermore, MetSyn is associated with an increased risk for CVD and acute ischemic/nonembolic stroke in elderly patients (7–9).

Men with the metabolic triad are at increased risk for CVD; their odds ratio (OR) is 5.9, compared with that of men with normal levels for two of the three components of the triad (10). Additionally, the results of the Quebec cardiovascular study indicated that men with an elevated proportion of cholesterol carried in LDL particles with diameters smaller than 25.5 nm are at increased risk for CVD (11–13). Furthermore, MetSyn patients commonly have increased levels of the atherogenic sdLDL particles, increased sdLDL cholesterol (sdLDL-C) concentrations, and decreased mean and peak LDL particle size compared with non-MetSyn individuals (14–16).

The aim of the present study was to investigate the presence of the HTGW phenotype in men and women with or without the MetSyn, as well as the association of the HTGW phenotype with the atherogenic metabolic triad, including sdLDL-C levels and mean and peak LDL particle size.

MATERIALS AND METHODS

Study population. Patients (n = 260) attending the Outpatient Lipid Clinic of the University Hospital of Ioannina participated in the present study. The diagnosis of the MetSyn was based on the NCEP ATP III criteria (5). Blood pressure was measured in the sitting position after a 5-min rest. Two consecutive measurements were carried out with an interim of 3 min, and the

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Abbreviations: BMI, body mass index; CHD, coronary heart disease; CVD, cardiovascular disease; HTGW, hypertriglyceridemic waist; MetSyn, metabolic syndrome; sdLDL-C: small, dense LDL cholesterol; NCEP ATP III, National Cholesterol Education Program Adult Treatment Panel III; PPD, peak particle diameter; TC, total cholesterol.

mean value was used. Waist circumference was measured midway between the iliac crest and the lowest rib.

Exclusion criteria were: prior atherosclerotic disease (myocardial infarction, unstable angina, ischemic stroke, peripheral arterial disease, percutaneous transluminal coronary angioplasty, or coronary artery bypass graft), known diabetes mellitus (fasting glucose ≥ 126 mg/dL), liver disease (serum aminotranferase activity greater than threefold, i.e., > 120 IU/L for normal range 5–40 IU/L), impaired renal function (serum creatinine levels greater than 1.5 mg/dL, normal range 0.6–1.2 mg/dL), or hypothyroidism (TSH greater than 5 μIU/mL, normal range 0.5–4.8 μIU/mL). Moreover, patients receiving drugs that could affect lipid metabolism as well as renal or hepatic function were excluded.

The HTGW phenotype was defined differently in men and women: waist circumference ≥ 90 cm and TG levels ≥ 180 mg/dL (1) for men, and waist circumference ≥ 88 cm and TG levels ≥ 150 mg/dL for women (2). The cutoff points for apolipoprotein (apo) B and insulin for the definition of the metabolic triad were derived from the median values of these parameters in non-obese, defined as body mass index (BMI) < 25 kg/m^2, men (n = 50) and women (n = 50) who did not fulfill any of the MetSyn criteria or the criteria of the HTGW phenotype, as described above. A mean LDL particle size cutoff point (26.8 nm) was used for both men and women, according to the Lipoprint LDL System instructions (see LDL subclass analysis section) (17). Because of the different reference range for apo B and insulin in men and women, the metabolic triad was defined separately: apo B > 96 mg/dL, insulin > 8 mU/L, and mean LDL particle size ≥ 26.8 nm for men; and apo B > 85 mg/dL, insulin > 6.85 mU/L, and mean LDL particle size ≥ 26.8 nm for women. The Ethics Committee of the University Hospital of Ioannina approved the study and every participant gave his/her written consent.

Biochemical parameters. All biochemical determinations were carried out after an overnight fast (approximately 12 h). Serum total cholesterol (TC), HDL cholesterol (HDL-C), and TG were determined enzymatically with an Olympus AU600 automated analyzer (Olympus Diagnostica, Hamburg, Germany). Serum LDL-cholesterol (LDL-C) levels were calculated using the Friedewald formula (provided that TG levels were lower than 400 mg/dL). In patients with TG levels greater than 400 mg/dL (n = 11) the LDL-C concentration was not recorded. Serum apo A-I and B were measured with a Behring Holding Gmbh analyzer (Liederbach, Germany). Serum insulin levels were measured by microparticle enzyme immunoassay on an AxSYM analyzer (Abbott Diagnostics). The homeostasis model assessment (HOMA) index was calculated as follows: fasting insulin (mU/L) × fasting glucose (mg/dL)/405.

LDL subclass analysis. Electrophoresis was performed using high-resolution 3% polyacrylamide tube gel and Lipoprint LDL System (Quantimetrix, Redondo Beach, CA) according to the manufacturer's instructions (17,18). Briefly, 25 μL of sample was mixed with 200 μL of Lipoprint Loading Gel and placed on the upper part of the 3% polyacrylamide gel. After 30 min of photopolymerisation at room temperature, electrophoresis was performed for 60 min with 3 mA for each gel tube. Each electrophoresis chamber involved two quality controls (sample provided by the manufacturer). For quantification, scanning was performed with a ScanMaker 8700 digital scanner (Mikrotek Co.) and iMac personal computer (Apple Computer Inc.). After scanning, electrophoretic mobility (Rf) and the area under the curve (AUC) were calculated qualitatively and quantitatively with the Lipoprint LDL system Template and the Lipoware software (Quantimetrix Co., Redondo Beach, CA), respectively. The LDL subfraction was calculated using the Rf between the VLDL fraction (Rf 0.0) and the HDL fraction (Rf 1.0). LDL is distributed from Rf 0.32 to Rf 0.64 as seven bands, whose Rf values are 0.32, 0.38, 0.45, 0.51, 0.56, 0.6, and 0.64 (LDL1 to LDL7, respectively). LDL1 and LDL2 are defined as large, buoyant LDL, and LDL3 to LDL7 are defined as sdLDL. The cholesterol concentration (in mg/dL) of each LDL subfraction is determined by multiplying the relative area (AUC) of each subfraction by the TC concentration of the sample (the TC concentration of the sample is measured independently). The proportion of sdLDL-C (sdLDL%) was defined as the percentage of the LDL-C carried in sdLDL (i.e., bands 3 to 7). LDL peak particle diameter (LDL-PPD) (nm) was determined using the Rf of the highest peak of the LDL bands according to the following equation proposed: LDL-PPD = (1.429-Rf) × 25 (19). Moreover, the Lipoprint LDL System provides a mean LDL particle size (nm) and uses a size of 26.8 nm as a cutoff point to classify individuals into phenotypes A (absence of sdLDL particles) and non-A (presence of sdLDL particles).

Statistical analysis. Preliminary analysis was performed to ensure no violation of the assumptions of normality and linearity. The Shapiro-Wilk test was used to evaluate whether each variable followed a Gaussian distribution. Data are expressed as mean ± SD, except for parameters not following a Gaussian distribution, which are expressed as median (range). The relationships between study parameters were assessed using the Pearson product-moment correlation coefficient (r) or Spearman's rank order correlation (rho) as appropriate. Independent samples t-test (or Mann-Whitney U test when appropriate) was used to assess differences between HTGW (+) and HTGW (−) individuals. One-way ANOVA or the Kruskal-Wallis test was used to explore differences between Gaussian and non-Gaussian results, respectively, according to waist circumference and TG levels. The Chi-square test (with Yates's correction) was used to assess the differences in the proportion of subjects characterized by the metabolic triad.

The sensitivity of HTGW phenotype to identify individuals with increased levels of sdLDL-C or decreased mean LDL particle size was calculated using the following equation:

[True positives/(true positives + false negatives)]*100

where true positives is the number of patients with sdLDL-C levels equal to or greater than 6 mg/dL or mean LDL particle size less than 26.8 nm correctly classified by the HTGW phenotype and false negatives is the number of patients with

sdLDL-C levels equal or greater than 6 mg/dL or mean LDL particle size less than 26.8 nm misclassified by HTGW phenotype. The specificity of the previously mentioned phenotype was calculated using the following equation:

[True negatives/(true negatives + false positives)]*100

where true negatives is the number of patients with sdLDL-C levels less than 6 mg/dL or mean LDL particle size greater than or equal to 26.8 nm correctly classified by HTGW phenotype and false positives is the number of patients with sdLDL-C levels less than 6 mg/dL or mean LDL particle size greater than or equal to 26.8 nm misclassified by phenotype. The positive and negative predictive values were calculated as [true positives/(true positives + false positives)]*100 and [true negatives/(true negatives + false negatives)]*100, respectively. A P value of less than 0.05 was considered to be significant. All analyses were carried out with the SPSS 13.0 softpack.

RESULTS

Of the total cohort, 133 individuals (53 men and 80 women) fulfilled the NCEP ATP III criteria for the diagnosis of the MetSyn, and 127 (52 men and 75 women) did not. Of the MetSyn subjects, 66.2% presented with the HTGW phenotype (n = 88), whereas the corresponding proportion in the non-MetSyn group was 5.5% (n = 7).

Table 1 shows the clinical and biochemical characteristics of the total cohort as well as of the men and women separately. In the overall cohort, HTGW (+) subjects were older than HTGW (–) individuals, and this was due to the difference in age in women. By definition, HTGW (+) subjects across all study groups had greater waist circumference compared with HTGW (–) individuals. Moreover, HTGW (+) subjects had higher systolic and diastolic blood pressure in the overall cohort and in women compared with HTGW (–) subjects, but blood pressure did not differ between HTGW (+) and HTGW (–) men. Finally, HTGW (+) individuals had a more adverse glycemic profile with elevated fasting glucose and insulin levels, as well as increased HOMA index compared with HTGW (–) subjects; this finding was consistent across all study groups.

Table 2 shows the lipid and lipoprotein profile of the study participants. In the overall cohort, HTGW (+) subjects had increased levels of TC, TG, non-HDL cholesterol, apo B, and the atherogenic index apo B/apo A-I ($P < 0.001$ for all comparisons), as well as decreased concentrations of HDL-C and apo A-I ($P < 0.001$ for both) compared with HTGW (–) individuals. LDL-C levels did not differ between the two groups. In men, the same pattern was observed with the exception of TC levels, which were similar in HTGW (+) and HTGW (–) individuals. In women, LDL-C levels were higher in the HTGW (+) group compared with the HTGW (–) group ($P < 0.05$).

The HTGW (+) group had higher concentrations of VLDL-C, sdLDL-C, and sdLDL% compared with HTGW (–) subjects ($P < 0.001$ for all comparisons) (Table 2). Moreover, HTGW (+) patients had significantly lower mean LDL particle size and LDL-PPD ($P < 0.001$ for all comparisons) compared with HTGW (–) individuals, and this finding was present in the overall cohort, as well as in the male and female subpopulations (Table 2).

Because menopause is associated with a body fat redistribution (20), we analyzed pre- and postmenopausal women separately to establish whether the differences in LDL subfractions were maintained. Indeed, the waist circumference in the HTGW (–) women was significantly greater in the postmenopausal group (100 vs. 89 cm, $P = 0.001$). However, a similar difference in the HTGW (+) women was not significant (111 vs. 104 cm), possibly reflecting the small number of HTGW (+) premenopausal women (n = 11). The differences in LDL characteristics between HTGW (+) and HTGW (–) were similar in pre- and postmenopausal women. Thus, in premenopausal women, comparing HTGW (–) (n = 63) with HTGW (+) (n = 11) groups: sdLDL-C, 3 (0–42) vs. 11 (0–51) mg/dL, $P = 0.02$; sdLDL%, 2.9 (0–41.2) vs. 9.9 (0–37.2), $P = 0.02$; LDL-PPD, 28.0 (25.0–29.0) vs. 26.7 (25.7–28.7) nm, $P = 0.03$; mean LDL particle size, 27.0 (24.9–27.5) vs. 26.4 (25.2–27.4) nm, $P = 0.008$. In postmenopausal women, comparing HTGW (–) (n = 38) with HTGW (+) (n = 43) groups: sdLDL-C, 5 (0–40) vs. 21.5 (1–79) mg/dL, $P < 0.001$; sdLDL%, 4.0 (0–22.1) vs. 15.8 (0.8–51.4), $P < 0.001$; LDL-PPD, 28.0 (26.0–28.7) vs. 26.6

TABLE 1
Clinical and Biochemical Characteristics of the Study Population[a]

	Total cohort (n = 260)		Men (n = 105)		Women (n = 155)	
	HTGW (+) (n = 95)	HTGW (–) (n = 165)	HTGW (+) (n = 41)	HTGW (–) (n = 64)	HTGW (+) (n = 54)	HTGW (–) (n = 101)
Age (yr)	53.2 ± 11.6	47.0 ± 11.2[a]	49.0 ± 10.9	47.7 ± 12.1	56.4 ± 11.1	46.5 ± 10.7[a]
Waist circumference (cm)	110 ± 12	97 ± 16[a]	110 ± 11	103 ± 12[b]	110 ± 13	93 ± 17[a]
SBP (mm Hg)	140 ± 17	129 ± 18[a]	135 ± 17	133 ± 19	144 ± 16	127 ± 16[a]
DBP (mm Hg)	89 ± 9	83 ± 11[a]	87 ± 9	84 ± 11	90 ± 9	82 ± 11[a]
Fasting glucose (mg/dL)	103 ± 12	97 ± 12[a]	106 ± 12	99 ± 12[b]	100 ± 12	95 ± 13[a]
Insulin (mU/L)	13.3 (4.4–59.5)	8.5 (2.0–56.8)[a]	12.2 (4.4–59.5)	8.9 (3.7–56.8)[c]	14.3 (4.8–47.5)	8.4 (2.0–38.3)[a]
HOMA index	3.2 (0.4–18.2)	1.9 (0.4–19.8)[a]	3 (1.1–18.2)	2.2 (0.7–19.8)[c]	3.2 (0.4–12.0)	1.9 (0.4–11.8)[c]

[a]Statistics are as follows: [a]$P < 0.001$, [b]$P < 0.01$ and [c]$P < 0.05$ compared with the corresponding HTGW (+) group. HTGW: Hypertriglyceridemic waist, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, HOMA: Homeostasis Model Assessment.

TABLE 2
Lipid and Lipoprotein Profile of the Study Population[a,b]

	Total cohort (*n* = 260)		Men (*n* = 105)		Women (*n* = 155)	
	HTGW (+) (*n* = 95)	HTGW (–) (*n* = 165)	HTGW (+) (*n* = 41)	HTGW (–) (*n* = 64)	HTGW (+) (*n* = 54)	HTGW (–) (*n* = 101)
TC (mg/dL)	245 ± 44	220 ± 39[a]	233 ± 42	219 ± 38	255 ± 44	221 ± 40[a]
TG (mg/dL)	233 (150–776)	100 (36–376)[a]	297 (183–776)	110 (45–179)[a]	208 (150–485)	94 (36–376)[a]
HDL–C (mg/dL)	49 ± 11	56 ± 12[a]	42 ± 8	51 ± 9[a]	54 ± 9	59 ± 12[b]
LDL–C (mg/dL)	148 ± 38	144 ± 33	134 ± 33	146 ± 31	158 ± 38	143 ± 33[b]
nonHDL–C (mg/dL)	197 ± 39	165 ± 36[a]	192 ± 37	169 ± 35[a]	201 ± 40	162 ± 36[a]
Apo A–I (mg/dL)	132 ± 24	143 ± 27[a]	126 ± 21	138 ± 20[b]	137 ± 26	147 ± 30[b]
Apo B (mg/dL)	112 ± 28	99 ± 22[a]	117 ± 25	103 ± 23[b]	109 ± 30	97 ± 30[b]
Apo B/Apo A–I	0.85 (0.41–1.79)	0.68 (0.32–1.33)[a]	0.91 (0.51–1.41)	0.71 (0.39–1.17)[a]	0.82 (0.41–1.79)	0.65 (0.32–1.33)[a]
Lp(a) (mg/dL)	10 (2–102)	10 (2–113)	9 (2–49)	9 (2–113)	10 (2–102)	10 (2–94)
VLDL–C (mg/dL)	56 (26–143)	38 (10–84)[a]	64 (26–143)	40 (16–75)[a]	56 (34–103)	37 (10–84)[a]
sdLDL–C (mg/dL)	22 (0–79)	5 (0–50)[a]	25 (4–79)	7 (0–50)[a]	21 (0–79)	4 (0–42)[a]
sdLDL (% of total LDL–C)	16 (0–56)	4 (0–41)[a]	23 (3–56)	5 (0–33)[a]	15 (0–51)	4 (0–41)[a]
LDL peak particle diameter (nm)	26.5 (23.7–29.0)	28.0 (25.0–29.0)[a]	26.2 (23.7–28.1)	27.7 (25.7–28.7)[a]	26.7 (23.7–29.0)	28.0 (25.0–29.0) [a]
Mean LDL particle size (nm)	26.1 (24.2–27.4)	26.9 (24.9–27.5)[a]	25.9 (24.2–27.0)	26.9 (25.5–27.5) [a]	26.2 (24.4–27.4)	27.0 (24.9–27.5) [a]

[a]Statistics are as follows: [a]$P < 0.001$ and [b]$P < 0.05$ compared with the corresponding HTGW (+) group.
[b]HTGW, hypertriglyceridemic waist; TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; Apo, apolipoprotein; Lp(a), lipoprotein (a); VLDL-C, VLDL-cholesterol; sdLDL, small, dense LDL.

(23.7–29.0) nm, $P < 0.001$; mean LDL particle size, 26.8 (25.9–27.4) vs. 26.2 (24.4–27.3) nm, $P < 0.001$.

Among all study participants, waist circumference correlated with HDL-C ($r = -0.23$, $P < 0.001$), nonHDL-C ($r = 0.32$, $P < 0.001$), TG (rho = 0.52, $P < 0.001$), apo B ($r = 0.24$, $P < 0.001$), apo B/apo A-I (rho = 0.35, $P < 0.001$), glucose ($r = 0.34$, $P < 0.001$), insulin (rho = 0.42, $P < 0.001$), and the HOMA index (rho = 0.46, $P < 0.001$). Moreover, waist circumference correlated with sdLDL-C and sdLDL% (rho = 0.32, $P < 0.001$ for both) as well as with mean LDL particle size and LDL-PPD (rho = –0.35 and rho = –0.3 respectively, $P < 0.001$ for both). TG levels were negatively correlated with HDL-C (rho = –0.36, $P < 0.001$), and positively correlated with non-HDL-C (rho = 0.56, $P < 0.001$), apo B (rho = 0.44, $P < 0.001$), apo B/apo A-I ratio (rho = 0.52, $P < 0.001$), glucose (rho = 0.3, $P < 0.01$), as well as with insulin levels and the HOMA index (rho = 0.43, $P < 0.001$ for both). As expected, TG levels were positively correlated with sdLDL-C and sdLDL% (rho = 0.61 and rho = 0.6, respectively, $P < 0.001$ for both) and were negatively correlated with mean LDL particle size and LDL-PPD (rho = –0.63 and rho = –0.6 respectively, $P < 0.001$ for both).

We divided the participants into three groups according to their waist circumference and into four groups according to their TG concentrations, separately for men and women (see Fig. 1 caption). Figures 1A and 1B show the distribution of sdLDL% and LDL-PPD across waist circumference and TG groups in men and women, respectively. In both men and women, sdLDL% increased and LDL-PPD decreased as waist circumference and TG levels increased ($P < 0.01$ and $P < 0.001$, respectively). sdLDL-C levels increased in both sexes with increasing waist circumference ($P < 0.01$) and TG concentrations ($P < 0.001$) (data not shown).

Table 3 shows the mean levels of apo B and median values of insulin and mean LDL particle size across groups by waist circumference and TG concentrations in men and women. In men, mean LDL particle size decreased ($P < 0.01$) and insulin and apo B levels increased ($P < 0.001$ and $P < 0.05$, respectively) as waist circumference increased. These relationships were not linear for insulin and for apo B in men. Mean LDL particle size decreased ($P < 0.001$) and insulin and apo B levels increased ($P < 0.05$ and $P < 0.001$, respectively) as TG levels increased. The pattern was the same for women (Table 4).

We evaluated the proportion of subjects with the MetSyn and/or the HTGW phenotype who also had the metabolic triad (Fig. 2). Among men, 52.3% of the MetSyn (+) individuals and 66.7% of the HTGW (+) individuals had the metabolic triad. Among women the corresponding percentages were 42.3% and 50.0%. Only 22.2% and 10.6% of the MetSyn (–) subjects (men and women, respectively) and 19.6% and 15.2% of the HTGW (–) subjects (men and women, respectively) presented with the atherogenic metabolic triad (Fig. 2). When we evaluated the proportion of patients with the metabolic triad who had both the MetSyn and HTGW criteria, the proportion was not significantly different from the comparison when only one of these criteria was used.

We estimated the sensitivity, specificity, and positive and negative predictive values of the HTGW phenotype to identify individuals with increased sdLDL-C levels or decreased mean LDL particle size. The sensitivity and the specificity of the HTGW phenotype to identify subjects with elevated sdLDL-C levels were 53.9% and 87.3%, respectively, whereas the corresponding values for the identification of patients with decreased mean LDL particle size were 58.5% and 88.5%, respectively. Moreover, the positive and negative predictive values of the HTGW phenotype to identify subjects with elevated sdLDL-C levels were 85.4% and 57.8%, respectively, and the corresponding values for the identification of patients with decreased mean LDL particle size were 85.4% and 64.9%, respectively.

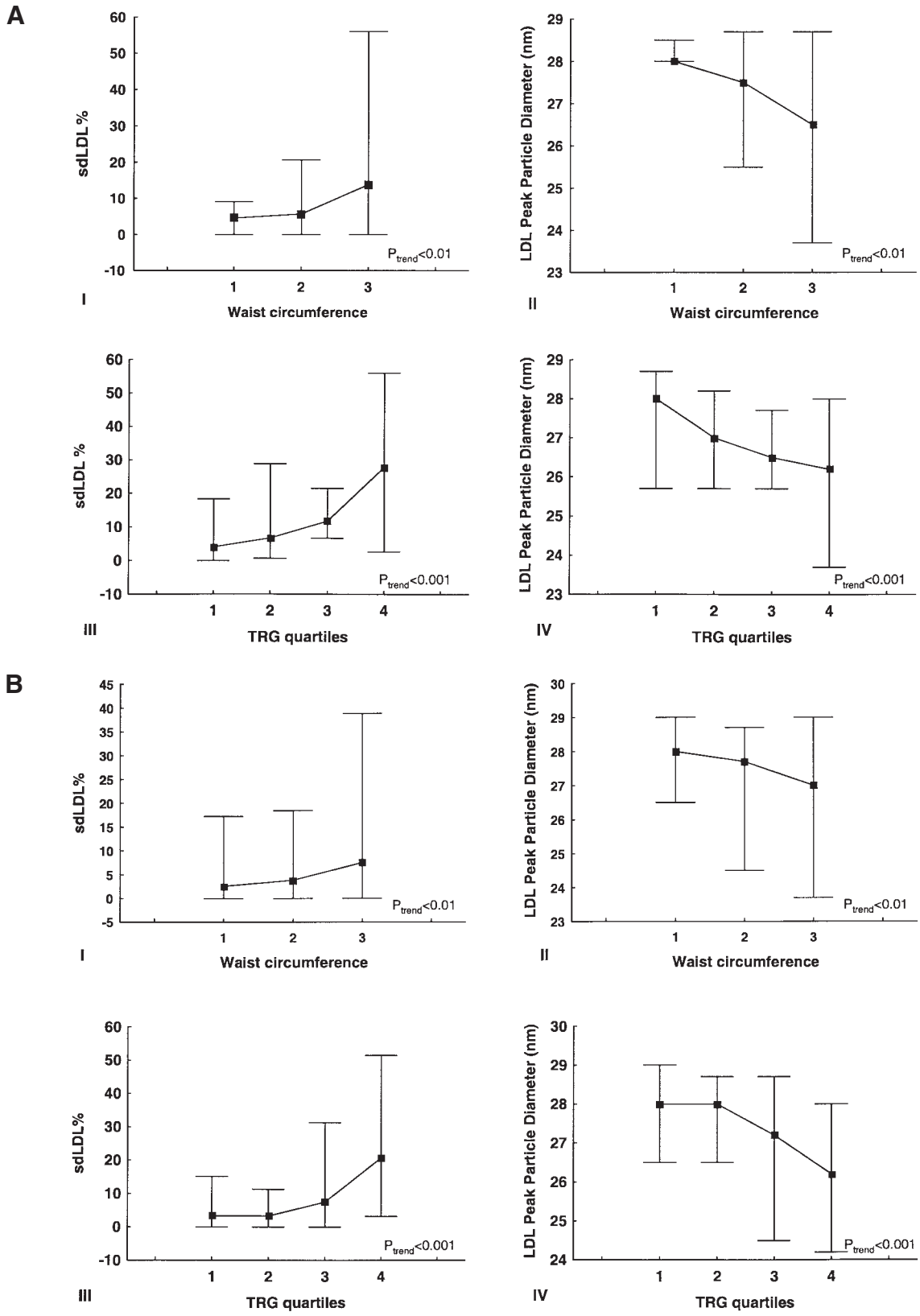

FIG. 1. (A) Small, dense LDL proportion and LDL peak particle diameter according to waist circumference and triglyceride levels in men. Waist circumference: Group 1, <90 cm; Group 2, 90–101 cm; Group 3, ≥102 cm. Triglyceride levels: Group 1: ≤149 mg/dL; Group 2, 150–179 mg/dL; Group 3, 180–200 mg/dL; Group 4, ≥201 mg/dL. (B) Small, dense LDL proportion and LDL peak particle diameter according to waist circumference and triglyceride levels in women. Waist circumference: Group 1, <88 cm; Group 2, 88–99 cm; Group 3, ≥100 cm. Triglyceride levels: Group 1, ≤130 mg/dL; Group 2, 131–149 mg/dL; Group 3, 150–200 mg/dL; Group 4, ≥201 mg/dL.

TABLE 3
Mean Levels of Apolipoprotein B (Apo B) and Median Values of Insulin and Mean LDL Particle Size According to Waist Circumference and TG Concentration in Men

	Apo B (mg/dL)	Insulin (mU/L)	Mean LDL particle size (nm)
Waist circumference			
1 (<90 cm)	91	11.3	27.0
2 (90–101 cm)	102	7.5	26.8
3 (≥102 cm)	113	12.1	26.2
	P for trend <0.05	*P* for trend <0.001	*P* for trend <0.01
TG levels			
1 (≤149 mg/dL)	97	8.0	26.9
2 (150–179 mg/dL)	119	11.4	26.6
3 (180–200 mg/dL)	104	8.7	26.4
4 (≥201 mg/dL)	120	12.6	25.6
	P for trend <0.001	*P* for trend <0.05	*P* for trend <0.001

TABLE 4
Mean Levels of Apolipoprotein B (Apo B) and Median Values of Insulin and Mean LDL Particle Size According to Waist Circumference and TG Concentration in Men

	Apo B (mg/dL)	Insulin (mU/L)	Mean LDL particle size (nm)
Waist circumference			
1 (<88 cm)	88	6.6	27.1
2 (88–99 cm)	104	10.7	26.9
3 (≥100 cm)	106	13.1	26.2
	P for trend <0.001	*P* for trend <0.001	*P* for trend <0.001
TG levels			
1 (≤130 mg/dL)	94	8.1	27.0
2 (131–149 mg/dL)	103	8.9	26.9
3 (150–200 mg/dL)	107	14.3	26.6
4 (≥201 mg/dL)	111	14.1	26.0
	P for trend <0.001	*P* for trend <0.05	*P* for trend <0.001

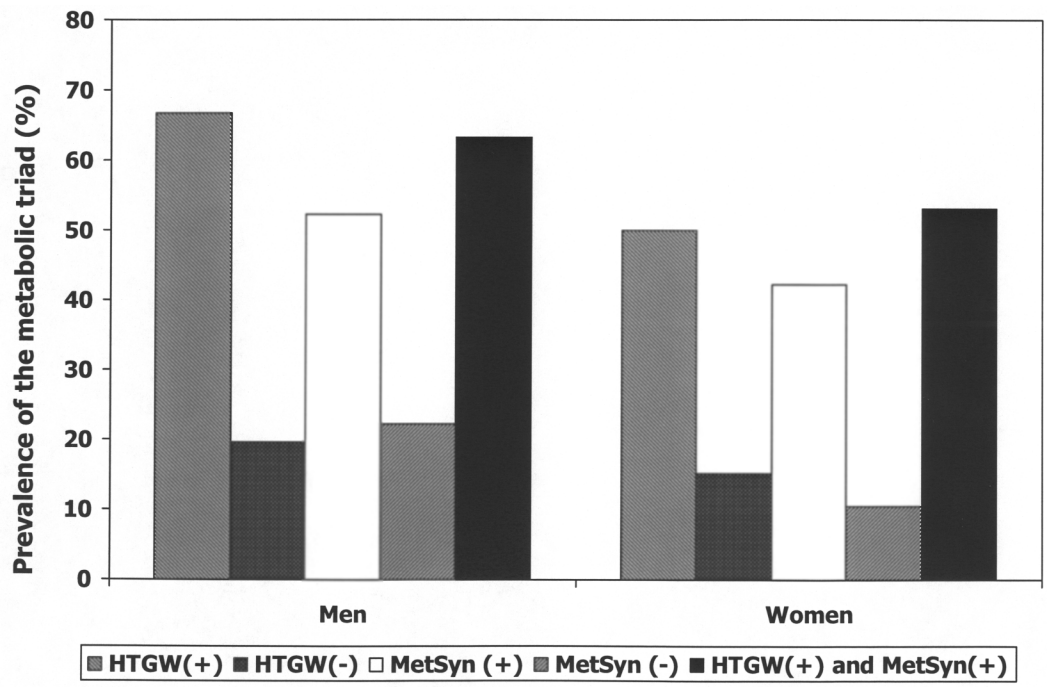

FIG. 2. Proportion of study participants with the metabolic triad. The metabolic triad was defined as mean LDL particle size < 26.8 nm, insulin > 8 mU/L for men and > 6.8 for women, and apolipoprotein B > 96 mg/dL for men and > 85 mg/dL for women.

DISCUSSION

The present study is to our knowledge the first to evaluate associations of HTGW (+) and MetSyn (+) with the amount of cholesterol carried in sdLDL particles and mean and peak LDL particle size.

Lemieux *et al.* in 2000 (1) were the first to propose that the HTGW phenotype is an inexpensive and sensitive marker of the atherogenic metabolic triad (characterized by hyperinsulinemia, hyperapolipoprotein B, and the presence of sdLDL particles). According to their results, about 80% of 185 apparently healthy men with a waist circumference ≥ 90 cm and TG levels ≥ 2 mmol/L (about 180 mg/dL) had the metabolic triad (1). According to our results, the corresponding proportion is similar at about 70%. However, we did not use as a cutoff point the median values of LDL-PPD as proposed by Lemieux *et al.* (1). Instead we used a cutoff point of mean LDL particle diameter less than or greater than 26.8 nm, as proposed by the manufacturer of the Lipoprint LDL system to identify individuals with phenotype A (mostly large, buoyant LDL particles) or non-A (presence of sdLDL particles) (17). Another study (2) showed that about 66% of HTGW (+) women had the metabolic triad. Our results show that among HTGW (+) women only 50% had the metabolic triad. A possible explanation for this discrepancy may be that in the other study (2) the LDL particle size was not recorded; it was substituted by LDL-C levels. In our study LDL-C levels in the female population showed only a weak correlation with mean LDL particle size (rho = −0.16, $P = 0.05$); thus, the metabolic triad in the study by La Monte *et al.* (2) might have been incorrectly estimated. Furthermore, in our study only 20% of HTGW (−) men and 15% of HTGW (−) women were characterized by the metabolic triad; the corresponding proportions in the studies by *Lemieux* (1) and LaMonte (2) were 10% in men and 22% in women.

The CVD risk factor profile tended to be worse in both HTGW (+) men and women compared with HTGW (−) individuals (Tables 1 and 2). This finding is in accordance with previous studies in both sexes (1,2,4,21). In the present study we evaluated more thoroughly the profile of the apo B-containing lipoproteins in HTGW (+) compared with previous studies. We found that HTGW (+) subjects were characterized by elevated concentrations of VLDL-C and sdLDL-C compared with HTGW (−) individuals (Table 2). Moreover, HTGW (+) patients had a decreased mean and peak LDL particle size compared with HTGW (−) individuals (Table 2). sdLDL% and sdLDL-C concentration increased and mean and peak LDL particle size decreased with increasing waist circumference and TG levels. The results of the Quebec Cardiovascular Study indicated that the amount of cholesterol carried in sdLDL particles predicts CVD risk even better than mean and peak LDL particle size (12). This finding gives more relevance to the present study which evaluated sdLDL-C levels in HTGW (+) individuals.

In our study menopause was associated with an increased waist circumference, and the effect was significant in the HTGW (−) group. Nevertheless, the difference in sdLDL-C, sdLDL%, as well as in mean and peak LDL particle size followed a similar pattern when comparing pre- and postmenopausal women with or without the HTGW phenotype.

The prevalence of the atherogenic metabolic triad was higher in HTGW (+) compared with MetSyn (+) subjects (66.7% vs. 52.3% in men, 50% vs. 42.3% in women, although these differences were not significant). The HTGW phenotype provides some degree of sensitivity and specificity for the identification of individuals with elevated sdLDL-C levels and decreased mean LDL particle size values.

In conclusion, HTGW (+) patients had an adverse metabolic profile characterized not only by altered concentrations of the classic lipid factors but also by disturbed apo B-containing lipoprotein profile. The HTGW phenotype provides an estimate of the presence of the atherogenic metabolic triad in adults of both sexes. Furthermore, the HTGW phenotype has the ability to recognize more subjects characterized by the metabolic triad than the presence of the MetSyn. This finding in addition to the fact that the diagnosis of the MetSyn requires more laboratory data than the diagnosis of the HTGW phenotype makes this a useful tool for the identification of individuals at increased risk for CVD.

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Soy Protein Containing Isoflavones Favorably Influences Macrophage Lipoprotein Metabolism but Not the Development of Atherosclerosis in CETP Transgenic Mice

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ABSTRACT: The possibility that soy protein containing isoflavones influences the development of experimental atherosclerosis has been investigated in ovariectomized mice heterozygous for the human CETP transgene and for the LDL-receptor null allele ($LDLr^{+/-}$ $CETP^{+/-}$). After ovariectomy at 8 wk of age they were fed a fat/cholesterol-rich diet for 19 wk and divided into three experimental groups: dietary unmodified soy protein containing isoflavones (mg/g of diet), either at low-dose (Iso Low, 0.272, $n = 25$), or at high-dose (Iso High, 0.535, $n = 28$); and the atherogenic diet containing an isoflavone-depleted alcohol-washed soy protein as a control group ($n = 28$). Aortic root lipid-stained lesion area (mean $\mu m^2 \times 10^3 \pm$ SD) did not differ among Iso Low (12.3 ± 9.9), Iso High (7.4 ± 6.4), and controls (10.7 ± 12.8). Autoantibody titers against plasma oxidized LDL did not differ among the experimental groups. Using the control mice as the reference value (100%), *in vitro* mouse peritoneal macrophage uptake of labeled acetylated LDL-cholesterol was lower in the Iso High (68%) than in the Iso Low (85%) group. The *in vitro* percent removal by exogenous HDL of labeled unesterified cholesterol from macrophages previously enriched with human [4-^{14}C]-cholesteryl oleate acetylated LDL was enhanced in the Iso High group (50%). In spite of these *in vitro* potentially antiatherogenic actions, soy protein containing isoflavones did not modify the average size of lipid-stained area in the aortic root.

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Although most retrospective epidemiological studies have indicated that hormonal replacement therapy effectively reduces coronary artery disease outcomes in postmenopausal women, recent large intervention trials have failed to demonstrate these beneficial effects (1–3). Due to the controversies surrounding conventional estrogen replacement therapy, soy-based food has been proposed as a dietary alternative to lower the plasma lipid concentration (4), in addition to improving the menopause-related symptoms (5,6) and osteoporosis (7). However, in spite of a suggestion that the intake of phytoestrogens has diminished the incidence of coronary heart disease in Japan (8) a definite proof of this benefit in Western societies is lacking (9–12). In addition, in humans, soy isoflavones have been shown to lower plasma lipids according to two meta-analyses (4,13), but not in two recent studies (14,15). Nonetheless, in several animal species, protection against the development of experimental atherosclerosis has been ascribed to phytoestrogens (16), soy isoflavones (17–20), a specific soy protein (21), and a soy-rich diet (22–24). However, on reviewing these studies, we noticed that in four (17,18,21,22), but not in four others (19,20,23,24), reductions of atherosclerosis and of plasma cholesterol occurred simultaneously. On the other hand, rabbits fed phytoestrogens have shown significantly reduced aortic cholesterol, an effect that the authors deem comparable to that of the estrogen replacement therapy (25). In addition to the fact that dietary soy cannot be strictly compared to isoflavone administration, it is worth noting that with the exception of some experiments involving moderate degrees of hypercholesterolemia (17,18,23), in all other experiments hypercholesterolemia attained by the experimental animals was remarkable or even extremely severe (16,18–22,24). Degrees of hypercholesterolemia attained are not ideal models for the effects of isoflavones in the human population, where plasma cholesterol concentrations are far more modest. Furthermore, these mentioned studies cannot strictly be compared because diets differed according to several components, such as soy, soy isolates, phytoestrogens, and pure isoflavones, making it difficult to interpret them in regard to their usefulness in humans. In spite of these considerations, several potentially beneficial actions of isoflavones concerning protection against premature atherosclerosis have been proposed, such as a reduction of LDL susceptibility to oxidation, improvement in vascular reactivity, and inhibition of pro-inflammatory cytokines, cell adhesion, and platelet aggregation (26,27).

We measured the effects of treatments with low and high doses of soy protein containing isoflavones on the development of aortic root lipid-laden lesions, and, considering the possible antiatherogenic mechanisms involved, we measured the formation of serum antibodies against oxidized LDL and the cholesterol uptake and efflux by mouse peritoneal macrophages *in vitro*. We utilized ovariectomized mice partially deficient in LDL receptor ($LDLr^{+/-}$), expressing a human CETP minigene ($CETP^{+/-}$), as previously reported

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(28,29). This model was chosen because the total cholesterol concentration was mild, well within the range of a normal human population, and human CETP was expressed.

EXPERIMENTAL PROCEDURES

Animals. The animal protocols were approved by the University of São Paulo Medical School Ethics Committee (protocol number 191/01). Female $LDLr^{+/-}$ $hCETP^{+/-}$ mice (n = 110) on a C57BL/6 background were obtained from the crossbreeding of female LDLr-knockout (from the Jackson Laboratory, Bar Harbor, ME) with male CETP-transgenic (a kind gift from Dr. H.C.F. Oliveira, Unicamp, Campinas, São Paulo, Brazil). Mice were weaned at 4 wk of age, and thereafter fed an *ad libitum* commercially available diet for 5 wk (Nuvital Nutrientes Ltda., Curitiba, PR, Brazil), housed 5 mice per cage and kept in a 12-h light-dark cycle in a conventional room. At 8 wk of age, they were ovariectomized, and 3 wk afterward their vaginal smears were collected to evaluate ovariectomy effectiveness. Only those animals that presented atrophic vaginal epithelium during a 5-d consecutive follow-up period were included. Animals were separated into three groups: those fed soy protein containing isoflavones at low dose (Iso Low, n = 25), those fed soy protein containing isoflavones at high dose (Iso High, n = 28), and controls fed isoflavone-depleted alcohol-washed soy protein (C, n = 28).

Diets and feeding procedures. Experimental diets were made utilizing the TD 88137 (21% milk saturated fat; 0.2% cholesterol by weight) supplied by Harlan Teklad (Madison, WI). All the experimental diets and the soy protein isolates (Clinical 670 Blend and FXP-H-0140) were a kind gift from Dr. Susan M. Potter (Solae Company, St. Louis, MO). The Clinical 670 Blend contained (mg/g product): genistein, 1.10; daidzein, 0.84; and glicytein, 0.20. The FXP-H-0140 component presented 0.04 mg/g of the product total aglycone. Although the FXP-H-0140 is an isoflavone-free ethanol-extracted product (AWISP), a diet totally devoid of isoflavones is not feasible; hence, insignificant amounts of isoflavones may be detected. The diet made with soy protein containing isoflavones at high dose (Iso High) was prepared using 250 g of Clinical 670 Blend, and the diet made with soy protein containing isoflavones at low dose (Iso Low) was prepared using 125 g of FXP-H-140 and 125 g of Clinical 670 Blend as the source of protein. The diet of the control mice was prepared with 250 g of FXP-H-140. In order to correct for the differences in protein content between FXP-H-140 and Clinical 670 Blend, small amounts (1.1% and 0.57%, respectively) of casein were added to Iso High and Iso Low diets. These diets did not differ regarding the proportions of calories that were represented as protein (19.4%), fat (42.0%), and carbohydrates (38.6%); diets are summarized in Table 1.

During the 19-wk study period, animals were fed the following amounts of isoflavones/100 g diet: 1 mg (C), 27 mg (Iso Low), and 53 mg (Iso High).

Lipid analysis. Plasma total cholesterol (TC) and triacylglycerol (TAG) concentrations were determined by enzymatic assays with commercially available kits (Boehringer Mannheim, Darmstadt, Germany, and Merck KgaA, Buenos Aires, respectively).

TABLE 1
Experimental Diet Composition (g/kg)

	Iso High	Iso Low	Control
Casein	11.49	5.74	0
Soy protein[a]	250	250	250
Free isoflavone	0.535	0.2725	0.01
Sucrose	289	302	305
Corn starch	150	150	150
Anhydrous milk fat	202	202	202
Cholesterol	1.52	1.52	1.52
Cellulose	50	50	50
Minerals	21.7	23.6	25.5
Vitamins	10	10	10
Ethoxyquin	0.04	0.04	0.04
Fat (%)	42.0	41.9	41.8
Carbohydrate (%)	38.6	38.8	38.9

[a]Iso High: Clinical 670 Blend; Iso Low: 50% Clinical 670 Blend plus 50% FXP-H-0140; C: FXP-H-0140.

Histological analysis of atherosclerotic lesions. Mice were anesthetized with ketamine (100 mg/kg, ip, Ketalar, Parke-Davis, São Paulo, Brazil) and xylazine (32 mg/kg, ip, Rompum, Bayer S.A., São Paulo, Brazil) and their hearts perfused *in situ* with phosphate-buffered saline (PBS) followed by 10% PBS-buffered formaldehyde, after which they were excised and fixed in 10% formaldehyde for at least 2 d. The hearts were then embedded sequentially in 5%, 10%, and 25% gelatin. Processing and staining were carried out according to Paigen *et al.* (30). Briefly, the heart was sectioned directly under and parallel to the axis of the atrial leaflets; the upper section was embedded in tissue-freezing medium (TBS, Triangle Biomedical Sciences, Durham, NC) and frozen. Because several other studies revealed a preference for the development of lesions in the aortic root, the segment chosen for analysis extended from beyond the aortic sinus up to the point where the aorta first becomes rounded and was expressed as the sum of the lesions in six 10-µm sections, 80 µm distant from each other in a total aortic length of 480 µm. The slides were stained with oil red O and counterstained with Harris's hematoxylin and with light green. The lipid-stained lesions were quantified as described by Rubin *et al.* (31) using the Image Pro Plus software (version 3.0) for image analysis (Media Cybernetics, Silver Spring, MD). The slides were read by an investigator blinded to the experimental groups.

Detection of antibodies to oxidized LDL. Antibodies against holo-oxidized LDL (oxLDL) or antibodies anti-apoB epitope derived from oxLDL (ApoB-D) were measured in mouse plasma by ELISA (28,32). ApoB-D is a 22-amino acid peptide from a region of ApoB-100 not accessible to trypsin. Polystyrene microtiter plates (Costar, Cambridge, MA) were coated with 50 µL of human oxLDL (7.5 µg/mL, 20 mM Cu^{2+}, for 24 h) or 50 µL/well of ApoB-D (1 µg/mL) and kept overnight at 4°C. The plates were blocked with gelatin 1% (Invitrogen Co., Carlsbad, CA, USA) at room temperature for 2 h. Plates were washed twice with PBS (100 µL). Plasma

samples (50 μL, 1:100) were added, and the plates were incubated for 2 h at 4°C, followed by washing with 1% Tween 20 in PBS. A peroxidase-conjugated goat anti-mouse IgG (Pharmigen, San Diego, CA, USA) (50 μL, 1:5.000 in PBS) was added, and after 1 h at room temperature, the plates were washed. Finally, 75 μL of substrate solution (250 mg of tetramethylbenzidine in 50 mL of DMSO, 10 μL of 30% H_2O_2, 12 mL of citrate buffer, pH 5.5) were added, and, after incubation at room temperature for 15 min, the reaction was stopped by adding 25 μL of 2.0 M sulfuric acid. The optical density (OD) was then measured in a microplate reader (Titertek Multiskan MCC/340P, model 2.20, Labsystems, Finland) at 450 nm.

Cell culture studies. Freshly resident mice peritoneal macrophages were harvested in PBS (0.8% NaCl, 0.06% Na_2HPO_4, 0.02% KCl, and 0.04% KH_2PO_4), pH 7.4. Pelleted cells obtained after centrifugation at 500 *g*, 4°C for 3 min were resuspended at a final concentration of 3×10^6 cells/mL in RPMI 1640 medium containing 20% (vol/vol) fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (0.1 mg/mL), (L-glutamine-penicillin-streptomycin, Sigma Chemical, St. Louis, MO, USA). An aliquot (0.5 mL) was transferred into 24-well tissue culture plates and incubated in a humidifier incubator (5% CO_2 atmosphere at 37°C). To remove non-adherent cells after a 2-h incubation period, each plate was washed twice with RPMI 1640 medium without FCS and used for subsequent experiments.

HDL-mediated cellular cholesterol efflux. Adhered mouse peritoneal macrophages were loaded with CE according to the method described by Brown and Goldstein (33). Briefly, macrophages were incubated in RPMI 1640 medium containing 2 mg/mL FA-free BSA in the presence of [^{14}C]cholesteryl oleate-labeled acetylated LDL ([^{14}C]CE-acLDL, 50 μg of protein/mL) for 24 h, at 37°C and washed once with DMEM (Dulbelcco's Minimum Essential Medium) containing antibiotics. [^{14}C]CE-acLDL–loaded macrophages were incubated for 6 h with DMEM containing 2 mg/mL BSA in the presence of human HDL (100 μg protein/mL) as cellular cholesterol acceptor, and the medium was drawn for radioactivity analysis (Ultima Gold) in the LS6000 Beckman Beta Counter. Cells were washed with PBS and dissolved in 0.2 N NaOH for the measurements of cell-associated radioactivity and protein. Efflux rate was defined as the amount of [^{14}C] unesterified cholesterol in the medium expressed as a percentage of total [^{14}C] in the medium plus cells. Blank values were obtained by the incubation of labeled cells in medium containing only 2 mg/mL BSA and no acceptor lipoprotein.

Acetylated LDL cholesteryl ether uptake by cells. Adhered macrophages were incubated in RPMI 1640 medium containing 10% (vol/vol) lipoprotein deficient human serum (3.5 mg protein/mL of medium) in the presence of acetylated LDL (34) labeled with [^{3}H]cholesteryl oleoyl ether ([^{3}H]COE LDL), 50 μg of protein/mL, for 6 h at 37°C in a humidified incubator (5% CO_2 atmosphere). At the end of the incubation, cells were washed with PBS and solubilized in 0.2 N NaOH for the measurement of the cell-associated radioactivity and protein content. Cellular [^{3}H]COE uptake was defined as the amount of ^{3}H in the cells expressed as a percentage of that offered to the cells per mg of cellular protein.

Cholesteryl ester transfer protein activity assay (CETP). Plasma CETP activity was measured by an exogenous method. Briefly, a mixture of human VLDL and LDL (200 μL, 200 mg cholesterol/dL) was incubated with pooled donor human HDL particles (50 μL, 40 mg cholesterol/dL) labeled with [4-^{14}C]-CE and mouse plasma (10 μL), as the source of CETP and 10 μL of Tris buffer, in a final volume of 300 μL. Blanks were prepared with Tris/saline/EDTA buffer (10 mM/140 mM/1 mM), pH 7.4, and control plasma from chow diet–fed C57BL/6 mice that do not express CETP. Incubations were carried out at 37° C for 2 h. The apoB containing lipoproteins were then precipitated with a 1.6% dextran sulfate/1 M $MgCl_2$ solution (1:1) and radioactivity measured in the remaining supernatant in a scintillation solution Ultima Gold (Eastman Kodak Co., Rochester, NY) in the LS6000 Beckman Beta Counter (Beckman Instruments, Palo Alto, CA). The percentage of 4-^{14}C CE transferred from [^{14}C]-CE-HDL to VLDL + LDL was calculated as: [1 – (sample radioactivity/control radioactivity)] × 100.

Hepatic CETP mRNA measurement: extraction of total RNA. The total mouse liver RNA was isolated using Trizol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of all RNA samples was evaluated on borate agarose gel electrophoresis after ethidium bromide staining. The reverse transcription was performed with Superscript II pre-amplification system (Invitrogen) from 1 μg of total RNA in a final volume of 20 μL.

Determination of hepatic CETP mRNA level. The hepatic mRNA expression of CETP was determined by RT-PCR using the mouse glyceraldeyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. The sequences of each primer pair (sense and antisense) used in the RT-PCR reaction were the following: human CETP (225 bp, GenBank accession no. NM_000078, 5′-CCA AGG TGA TCC AGA CCG-3′ and 5′-TGG TGT AGC CAT ACT TCA GGG-3′); and GAPDH (394 bp, GenBank accession no. M32599, 5'-CTG CAT CCA CTG GTG CTG-3′ and 5′- AGG GTT TCT TAC TCC TTG GAG G-3′). The RT-PCR reaction was performed in a DNA thermal cycler PTC-200 (MJ Research Inc., Watertown, MA, USA) using the reverse transcription product co-amplified in the presence of sense and antisense primers of GAPDH (7.5 pmol) and CETP genes (15 pmol) in PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.3), 200 mM of each dNTP, 5% DMSO, 2 U Taq DNA polymerase (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), in a final volume of 50 μL. The PCR conditions were 94°C for 4 min, and subsequently 28 cycles at 94°C for 1 min, 54°C for 90 s, 72°C for 2 min, and finally at 72°C for 10 min. The PCR products were then submitted to electrophoresis in 2% agarose gel, stained with ethidium bromide, and the band densities were analyzed by the software from Alpha Imager TM 1220 (Alpha Innotec Co., SanLeandro, CA, USA). The relative density of each sample was nor-

malized to the signal of GAPDH and was expressed as the sample/GAPDH ratio.

Lecithin cholesterol acyltransferase assay (LCAT). The LCAT-mediated cholesterol esterification reaction was measured using endogenous substrates (35). Briefly, mice plasma (20 μL) was incubated with [4-^{14}C]-free cholesterol at 37°C for 24 h, and the free cholesterol (4-^{14}C-FC) and cholesterol ester (4-^{14}C-CE) were separated by TLC for radioactivity measurement in the scintillation solution Ultima Gold in a LS6000 Beckman Beta Counter.

Statistical analysis. All comparisons were analyzed by statistical tests using the GraphPad Prism, version 3.0 for Windows (GraphPad Software Inc., San Diego, CA). Differences were considered significant when $P < 0.05$.

RESULTS

The amount of diet consumed did not differ among all groups throughout the study (3.0 ± 0.7 g/d). Iso Low, Iso High, and control groups gained weight from the basal to the final periods (Table 2). At the end of the study, Iso High animals were the heaviest of the three groups.

Plasma lipid and lipoprotein concentrations. Plasma lipid and lipoprotein concentrations were determined 2 wk after ovariectomy (basal values), and again at the end of the study (19th wk). At the basal period, there were no differences among the groups, and at the end of the study, plasma triglycerides levels of Iso Low group were higher than the other groups (Table 3).

The average areas of atherosclerotic lesion did not differ among Iso Low, Iso High, and control groups (Table 4). Because of this lack of difference, we investigated the percent distribution of the lesions according to their size in reference to the control group median size (8.1×10^3 μm^2) so as to define small lesions (below median) and large lesions (above median). We found that the number of mice with small and large lesions were roughly evenly distributed in the Iso Low group, but in the Iso High group, there was a distribution trend favoring the prevalence of smaller lesions: 70% of all mice in this group presented small-type lesions ($P = 0.085$ by κ^2 test).

Antibody titers against oxidized LDL or against an epitope of oxidized apolipoprotein B (ApoB-D) did not differ among the three experimental groups (Table 5).

When compared with the Iso Low and control groups, Iso High mice presented lower mouse peritoneal macrophage up-

TABLE 2
Basal and Final Body Weights[a]

Body weight (g)	Iso Low	Iso High	Control
Basal	18.8 ± 2.9 (25)	20.0 ± 3.2 (27)	19.8 ± 4.1 (27)
Final	27.4 ± 3.9[b] (25)	33.5 ± 7.7[b,c] (28)	29.5 ± 4.8[b] (28)

[a]Mean ± SD. Values in parenthesis represent the number of animals in each group. Statistical comparisons: ANOVA followed by Newman-Keuls multiple comparison test.
[b]Basal vs final, $P < 0.05$.
[c]Final weight of Iso High is greater than the other groups.

TABLE 3
Plasma Lipid and Lipoprotein Concentrations (mmol/L) at Basal and Final Periods[a]

	Iso Low	Iso High	Control
TC			
(Basal)	2.4 ± 0.3 (21)	2.2 ± 0.3 (25)	2.3 ± 0.3 (26)
(Final)	4.0 ± 0.8	4.0 ± 0.8	3.9 ± 0.8
VLDL-C			
(Basal)	0.7 ± 0	0.7 ± 0	0.7 ± 0
(Final)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
LDL-C			
(Basal)	0.8 ± 0	0.7 ± 0	0.8 ± 0
(Final)	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
HDL-C			
(Basal)	0.9 ± 0	0.8 ± 0	0.8 ± 0
(Final)	1.6 ± 0.2	1.6 ± 0.1	1.5 ± 0.2
n-HDL-C			
(Basal)	1.5 ± 0	1.4 ± 0	1.5 ± 0
(Final)	2.5 ± 0.2	2.4 ± 0.1	2.4 ± 0.2
TAG			
(Basal)	1.1 ± 0.5 (14)	1.3 ± 0.9 (17)	1.3 ± 0.6 (16)
(Final)	1.1 ± 0.8[b] (16)	0.7 ± 0.3 (22)	0.8 ± 0.3 (22)

[a]Values are means ± SD. Values in parentheses represent the number of animals in each group. Lipoprotein fractions were obtained by FPLC from mice plasma pools (n = 3). (Non) n-HDL-C = TC – HDL-C. Statistical comparisons: TC and TAG, ANOVA followed by Newman-Keuls multiple comparison test. VLDL-C, LDL-C, HDL-C, and n-HDL-C, Kruskal-Wallis, followed by Dunns multiple comparison test.
[b]$P < 0.05$, Iso Low differs from Iso High and control.

TABLE 4
Area of Aortic Atherosclerosis Lesion[a]

Iso Low ($n = 24$)	Iso High ($n = 24$)	Control ($n = 22$)
12.3 ± 9.9	7.4 ± 6.4	10.7 ± 12.8

[a]Values are means ± SD, μm$^2 \times 10^3$. Statistical comparison: ANOVA followed by Newman-Keuls multiple comparison test. No differences amongst the experimental groups

TABLE 5
Serum Antibodies Titers Against Oxidized LDL or Oxidized apoB-D Peptide Measured as Optical Densities at 450 nm[a]

	Iso Low (n = 8)	Iso High (n = 10)	Control (n = 13)
ox-LDL	0.76 ± 0.10	0.69 ± 0.16	0.62 ± 0.15
ApoB-D	0.68 ± 0.06	0.67 ± 0.14	0.61 ± 0.14

[a]Statistical comparisons: ANOVA followed by Newman-Keuls multiple comparison test. No differences among the experimental groups.

TABLE 6
Basal and Final Plasma CETP Activity (%) and Hepatic CETP mRNA Expression (%) at the End of the Study

CETP	Iso Low	Iso High	Control
Basal	22.0 ± 7.5 (21)	23.2 ± 7.4 (25)	20.9 ± 5.2 (26)
Final	42.8 ± 8.6[a] (21)	40.8 ± 8.0[a] (25)	43.1 ± 7.6[a] (25)
mRNA	96.3 ± 6.8[b] (19)	95.3 ± 6.3[b] (20)	100 (20)

[a]Paired student's t test: $P < 0.001$, basal vs final in the experimental groups. mRNA values were normalized to GAPDH mRNA content and expressed as relative values.
[b]ANOVA followed by Newman-Keuls multiple comparison test: Iso Low and Iso High lower than control group at $P < 0.05$.

take of [^{3}H]COE acetyl-LDL (68%), and higher ^{14}C-cholesterol efflux rate (50%) from macrophages preloaded with [^{14}C]CE acetyl-LDL (Fig. 1).

Although the hepatic CETP mRNA expression was lower in Iso Low and Iso High than in the control group, plasma CETP activity was not influenced by the isoflavone treatment (Table 6).

Lecithin cholesterol acyl transferase (LCAT), hepatic (HL), and peripheral lipoprotein lipase (LPL) activities did not differ among all the groups (data not shown).

DISCUSSION

Considering that in addition to plasma cholesterol and lipoprotein concentrations, several other factors play roles in the pathogenesis of atherosclerosis, in this work we investigated the effects of soy protein containing isoflavones on the cholesterol uptake and efflux by mouse peritoneal macrophages, levels of antibodies against oxidized LDL, and average size distribution of the lipid-laden area in the aortic root.

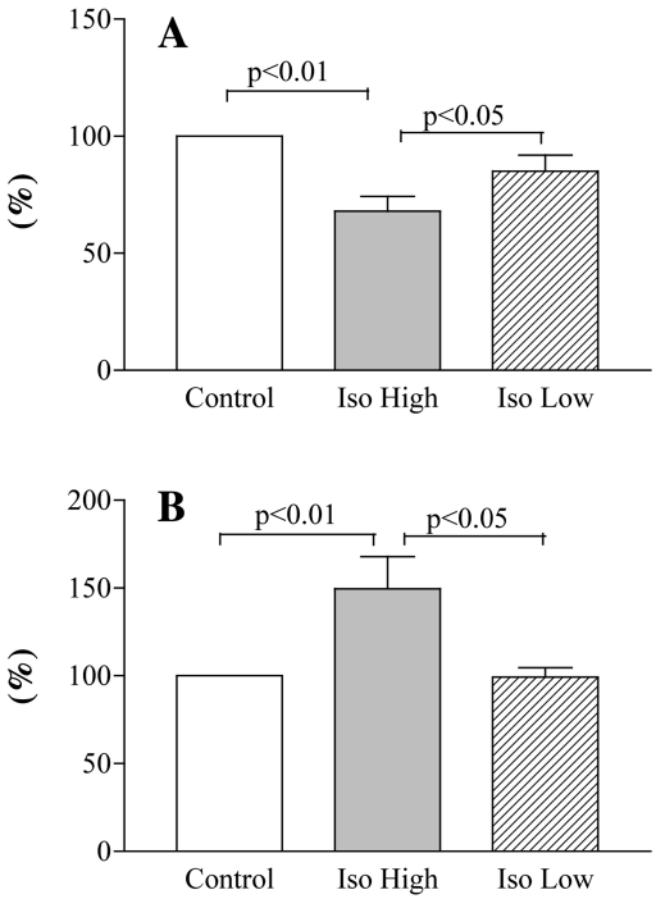

FIG. 1. Mouse peritoneal macrophage uptake of [^{3}H]COE acetyl-LDL (A) and HDL-mediated efflux of cellular unesterified ^{14}C-cholesterol (^{14}C present in the medium) in macrophages that had previously been loaded with [^{14}C]CE acetyl-LDL (B). Data are relative to the control group. Eight replicates of cell pools of from six to eight mice in each experimental condition are shown. Statistical comparison: ANOVA followed by Newman-Keuls multiple comparison test displayed in the figures.

Macrophages drawn from the Iso High group took up significantly less cholesterol and delivered into the culture medium significantly more of the internalized cholesterol than the Iso Low and control groups (Fig. 1). However, these results did not significantly modify the average size of lipid-stained area in the aortic root of isoflavone-treated mice (Fig. 2).

It is known that antibodies against ox-LDL are positively associated with the ox-LDL content in the atherosclerotic lesions in LDL receptor knockout mice (36), as well as with the severity of coronary arterial disease in humans (37). Nonetheless, the results remain controversial in humans; Heikkinen *et al.* (38) did not find differences in ox-LDL antibodies titers in postmenopausal women after one year on hormonal replacement, whereas Uint *et al.* (39) described increased anti-ox-LDL titers after 90 d on this treatment. In this regard, Wen *et al.* (40) and Santanam *et al.* (41) showed that, except for plasma concentrations over 7343 pmol/L, estrogens at physiological concentrations (499 to 4,993 pg/mL) do not protect LDL particles against oxidation. On the other hand, Damasceno *et al.* (32) found that rabbits fed soy protein containing isoflavones have lower autoantibodies against ox-LDL compared with casein-fed rabbits. However, in our study, antibodies against ox-LDL and against apoB-D did not differ among the experimental groups. Regarding the antioxidant action of isoflavones on LDL, although Samman *et al.* (42) did not find differences in the oxidation susceptibility of human LDL after isoflavones or placebo treatment, Meng *et al.* (27) described an increased lag time during the oxidation process, indicating resistance to oxidation likely due to the incorporation of esterified isoflavones into the LDL particles.

Measuring CETP activity in plasma was necessary in our study because plasma CETP inhibition has potentially antiatherogenic effects in humans and in experimental animals (43), but CETP activity was not influenced by the added isoflavones.

In general, estrogen treatment protects against aortic fat accumulation in rabbits, monkeys, and mice, as reviewed by Hodgin and Maeda (44). Nonetheless, in the majority of these investigations, including in our own, in transgenic mice expressing the human CETP (28,29), this protection seems to be related to the lowering of plasma total cholesterol in the non-HDL-C fraction, namely, in apoB-containing lipoproteins.

The present investigation showed that soy protein containing isoflavones resulted in (1) a trend for the formation of smaller lipid-laden aortic lesions, (2) impairment of the macrophage cholesterol loading, and (3) stimulation of HDL-mediated cellular cholesterol efflux, conferring a potentially antiatherogenic effect. Nonetheless, a cautionary note regarding our study concerns the fact that soy protein containing isoflavones and other alcohol isolated soy protein lower but do not eliminate the ability of soy protein to reduce atherosclerosis and thus as suggested by others may have influenced our results because alcohol-extracted soy protein was utilized in our investigation (45).

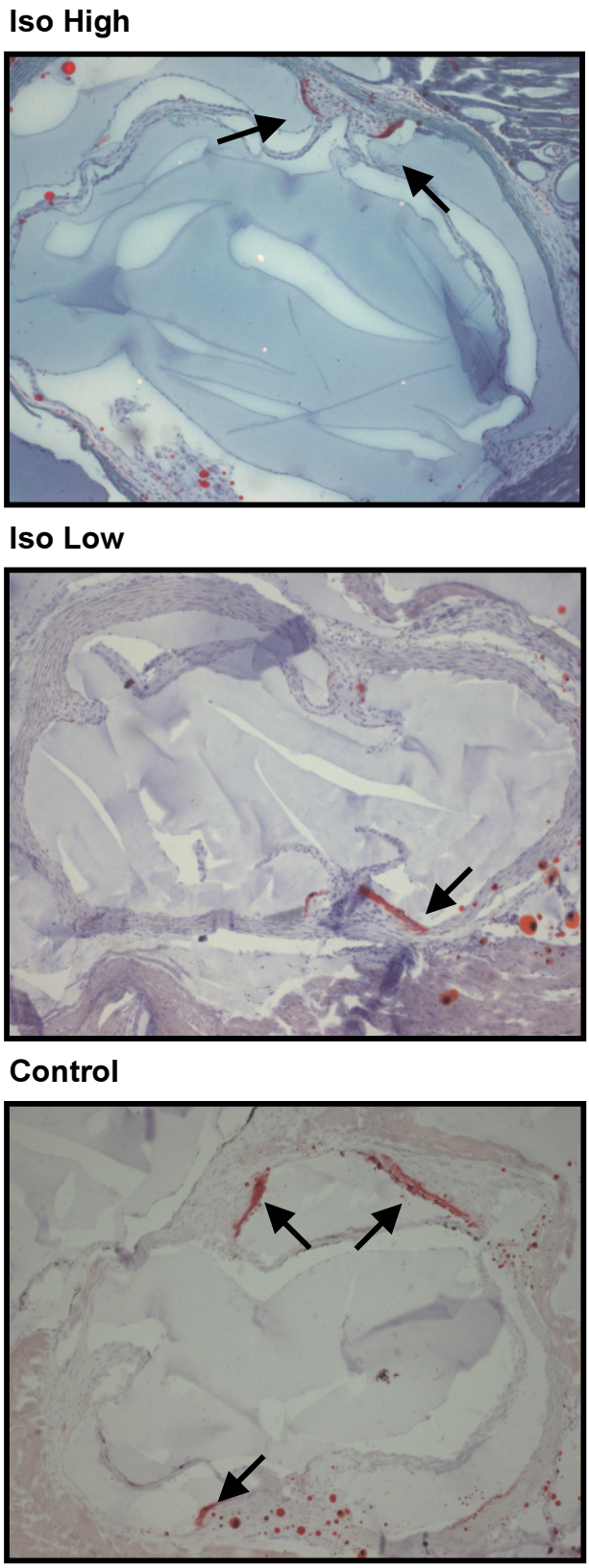

FIG. 2. Histological typical lesions stained with Oil red O and counterstained with Harris hematoxylin and with light green are indicated by arrows. Size increased 10 times.

Our study on the effects of soy protein containing isoflavones on atherosclerosis differs from other investigations in that we chose an experimental model where severe hypercholesterolemia was avoided. We then observed that soy protein containing isoflavones did not reduce the plasma cholesterol concentration or the degree of experimental atherosclerosis, a finding that agrees with another experimental study (23). On the other hand, in a few animal experiments where the degree of hypercholesterolemia was mild, soy isoflavone reduction or prevention of the development of the experimental atherosclerosis seemed consequent to the reduction of plasma cholesterol (17,18). However, in few studies where severe hypercholesterolemia was induced soy isoflavones protected against atherosclerosis without modifying the concentration of plasma cholesterol (19,20). Nonetheless, our study sheds light on the beneficial effects of soy protein containing isoflavones on the macrophage cholesterol metabolism that could have been more efficacious to protect against experimental atherosclerosis in the presence of severe hypercholesterolemia.

These effects need to be taken into account for the human use of this supplemental nutrient, considering that in humans the effects of isoflavones on plasma lipid concentration are controversial and that no trials have been published on the effects of isoflavones on cardiovascular disease (46,47).

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Diminished Macrophage Cholesterol Removal Rate by the Altered HDL Metabolism in the Nagase Analbuminemic Rat

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ABSTRACT: Dyslipoproteinemia of the Nagase analbuminemic rat (NAR) is characterized by elevated concentrations of VLDL and LDL attributed to increased rates of liver lipoprotein synthesis. Increased lysophosphatidylcholine (LPC) in NAR HDL has been attributed to high plasma LCAT activity. We show here that, as compared with Sprague-Dawley rats (SDR), NAR plasma triacylglycerol (TAG), total cholesterol (TC), HDL TAG, protein, total phospholipids (PL), LPC, and PS are increased. These alterations rendered the NAR HDL particle more susceptible to the activity of the enzyme hepatic lipoprotein lipase (HL), which otherwise was unaltered in our study. Fractional catabolic rates in blood of the autologous ^{125}I-apoHDL (median and lower quartile values), were, respectively, 0.231 and 1.645 (n = 10) in NAR as compared with 0.140 and 0.109 (n = 10) in SDR (P = 0.012), corresponding to synthesis rates of HDL protein of 89.8 ± 33.7 mg/d in NAR and 17.4 ± 6.5 mg/d in SDR (P = 0.0122). Furthermore, Swiss mouse macrophage free-cholesterol (FC) efflux rates, measured as the percent [^{14}C]-cholesterol efflux/6 h, were 8.2 ± 2.3 (n = 9) in NAR HDL and 11.2 ± 3.2 (n = 10) in SDR HDL (P = 0.03). Therefore, in NAR the modification of the HDL composition slows down the cell FC efflux rate, and together with the increased rate of plasma HDL metabolism influences the reverse cholesterol transport system.

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Hypoalbuminemia, proteinuria, hypercoagulability, edema, and dyslipoproteinemia that includes alterations of HDL composition and metabolism are common features of nephrotic syndrome (NS) (1). Understanding the mechanisms involved in this hyperlipidemia is critical to explain the increased cardiovascular risk in NS (2,3). Nephrotic syndrome animal models and the Nagase analbuminemic rat (NAR) share several alterations in the metabolism of lipoproteins (4).

From a stock of Sprague-Dawley rats (SDR), Nagase *et al.* (5) produced a strain of hyperlipidemic rats, NAR, completely deficient in serum albumin. The serum albumin deficiency is inherited as an autosomal recessive trait. Similar to NS, NAR hyperlipidemia has been characterized by increased total cholesterol (TC), TAG, free FA (FFA), and phospholipid (PL) plasma concentrations. Plasma apolipoproteins (apo) A-I, B, and E, as well as HDL lysophosphatidylcholine (LPC) are also significantly increased in NAR (6,7).

NAR total serum protein concentration is normal (5) but the plasma oncotic pressure is reduced (8). Although the relationship between low oncotic pressure and hyperlipidemia is not completely understood, previous studies have shown increased plasma TAG and VLDL synthesis rates in NAR that explain the pathogenesis of hyperlipidemia in NS (9,10).

In analbuminemia, substantial amounts of FFA and LPC (6,7) are bound to lipoprotein (LP) fractions, whereas in normal animals albumin is the main carrier of these components (11,12). Higher LPC concentration in NAR erythrocytes is normalized after albumin addition to blood (13), and monolayer cell permeability of human coronary artery endothelial cells is increased in a time- and dose dependent manner with LPC treatments (14). Furthermore, albumin protects endothelium-dependent relaxation against the inhibitory effect of LPC (15) and reduces LPC uptake by cultured endothelial cells (16). Thus, the role of albumin as a reservoir for LPC may be important to maintain the integrity of the monolayer endothelial cell. Injury or dysfunction of the monolayer endothelial cell is considered one of the critical events in the development of atherosclerosis (14).

Although rats are not suitable models for studies of atherosclerosis (17), NAR and human analbuminemia (5) are useful tools for understanding whether alterations in the metabolism of HDL play a role in the regulation of the body cholesterol homeostasis that may explain the mechanisms of premature atherosclerosis in humans. Thus, the current study aims at investigating whether the anti-atherogenic role of HDL is impaired by modifications of the HDL composition due to alterations of HDL metabolism in NAR.

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Abbreviations: apo, Apolipoprotein; ^{14}C-PC, L-dipalmitoyl [choline-methyl-^{14}C] PC; DMEM, Dulbecco's modified Eagle`s medium; EDTA-PBS, ethylenediaminetetraacetic acid phosphate-buffered saline; FBS, fetal bovine serum; FC, free cholesterol; FCR, fractional catabolic rate; FAFA, fatty acid free albumin; FFA, free FA; HL, hepatic lipase; ^{125}I-apoHDL, radioactive HDL; LP, lipoprotein; LPC, lysophosphatidylcholine; NAR, Nagase analbuminemic rat; NS, nephrotic syndrome; PL, phospholipid; SDR, Sprague-Dawley rats; SM sphingomyelin; SR-BI, scavenger receptor B type I; RCT, reverse cholesterol transport system; TC, total cholesterol; TCA, trichloroacetic acid.

EXPERIMENTAL PROCEDURES

Animals. Breeding NAR and SDR colonies were kindly supplied by Professors Roberto Zatz and Clarice K Fujihara (Renal Division of University of São Paulo Medical School). Animals were raised and fed *ad libitum* a pelleted commercial chow (Nuvilab-Nuvital, São Paulo, Brazil) in conventional housing at 22 ± 2°C with a light-dark cycle of 12 h. Animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee (nos. 582/01 and 029/02). In order to avoid the interference of chylomicron TAG on plasma total measurements, all experiments were carried out in the morning after a 12-h overnight fasting period. Under ketamine (60 mg/kg body weight, i.p.) and xylazine (10 mg/kg body weight, i.p.) anesthesia, the right carotid and abdominal aorta arteries of 3-mon-old male NAR and SDR were cannulated with indwelling polyethylene catheters PE-50 and PE-100 (Intramedic, Clay Adams, Parsippany, NJ), respectively, previously rinsed with liquid silicon (Silicone Prontosil, Rio de Janeiro, Brazil). The right carotid artery isolation was carried out by a small incision in the ventral median line of the neck, and the abdominal aorta artery was cannulated after a median abdominal laparotomy. All experiments started after the animals had completely recovered from surgery. At the end of metabolic studies rats were sacrificed in a CO_2 chamber.

Isolation and labeling of HDL. Blood was drawn over heparin (20 IU, 5μL) by exsanguination from the rat abdominal aorta (NAR, $n = 12$; SDR, $n = 9$). HDL ($d = 1.063$–1.21 g/mL) was separated from plasma by sequential ultracentrifugation (L-80 ultracentrifuge, Beckman Instruments, Palo Alto, CA) in a 50Ti rotor after spinning at $d > 1.21$ g/mL, 200 000 × g, for 40 h at 4°C as previously described (18). HDL purification was performed by discontinuous gradient ultracentrifugation (19) utilizing an SW 41 rotor spinning at 200 000 × g for 24 h at 4°C. LP was dialyzed against EDTA-PBS. The protein content was determined according to Lowry *et al.* (20). HDL LPC, sphingomyelin (SM), PC, PS, and PE fractions were separated by TLC (21) and measured by the modified micro-procedure of Bartlett (22). TC and TAG were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany). Fresh NAR ($n = 12$) or SDR ($n = 9$) HDL (500 μL) was incubated with 17 μl of L-dipalmitoyl [choline-methyl-^{14}C] PC (^{14}C-PC) (Dupont-New England Nuclear, Boston, MA) and their respective infranatants (3 mL; $d > 1.21$ g/mL) in a shaking water bath for 18 h at 37°C (23). The purity of the isotope was checked by silica gel TLC and shown to be higher than 98%. NAR and SDR ^{14}C-PC-HDL were then re-isolated by sequential ultracentrifugation as described above.

Hepatic post-heparin plasma lipolytic activity. Four minutes after intra-carotid heparin injection (100 IU/kg body weight), NAR ($n = 3$) and SDR ($n = 3$) post-heparin plasma lipases were obtained from the abdominal aorta. This time was selected based upon the time-course of appearance of lipolytic activities (24), and the hepatic lipase (HL) activity was determined after inhibition of the peripheral lipoprotein lipase activity as previously described (25). Nonetheless, the measurement of HL at a single time point does not reflect the HDL TAG hydrolysis rate over time. Briefly, NAR and SDR HDL were incubated with Tris-HCl (2 M NaCl, pH 8.0; Sigma Chemical Co., St Louis, MO) using autologous and heterologous post-heparin plasma in a shaking bath for 1 h at 37°C. Folch (3 mL chloroform/methanol, 2:1, vol/vol; E. Merck, Darmstadt, Germany) was added to the samples at the end of incubation. Following an overnight period at 4°C, the infranatant was then evaporated under N_2 flow. After separation by TLC, radioactivity counts in PC and LPC bands were determined in a beta scintillation counter (LS 6000-TA8, Beckman Instruments). HL activity was estimated as the percentage conversion of PC to LPC.

Iodination and kinetics of HDL. After dialysis, NAR HDL (3.69 mg/dL) and SDR HDL (1.61 mg/dL) protein pools were iodinated with iodine monochloride (Sigma Chemical Co.) and sodium iodide I-125 (Na-^{125}I) (MDS Nordion, Kanata, Ontario, Canada) (26). Radioactive HDL (^{125}I-apoHDL) pools were exhaustively dialyzed against PBS (pH 7.4), and an aliquot precipitated by TCA (E. Merck). More than 95% of ^{125}I-apoHDL was precipitated by TCA.

Four days before as well as during the ^{125}I-apoHDL kinetic study, animals had free access to drinking water containing potassium iodide and iodine (1.5 mg in each 500 mL) to avoid ^{125}I uptake by the thyroid. Before and after the experiment, blood samples (70 μL) were drawn for hematocrit, TAG, and TC measurements. Blood samples (100 μL) were sequentially drawn from the tail vein over a period of 48 h (at 3 min, 10 min, 6 h, 10 h, 24 h, 32 h, and 48 h) after an intra-carotid autologous infusion of NAR or of SDR ^{125}I-apoHDL. For this purpose rats were lightly restrained but not anesthetized. Radioactivity was measured in 40-μL plasma aliquots in a gamma counter (Cobra Model, Packard Instruments, Meriden, CT). Rats were normally fed 3 h after the ^{125}I-apoHDL infusion. Plasma ^{125}I-apoHDL clearance curves were analyzed as the fractional catabolic rate (FCR), according to a compartmental analysis of the plasma protein radioactivity vs. time. The system is based on a two-pool model that assumes the existence of an intravascular pool in dynamic equilibrium with an extravascular pool and that new input or exit of radiolabeled HDL occurs from the intravascular pool only (27). FCR values were derived directly from the kinetic model as the clearance rate from the intravascular pool. Biexponential ^{125}I-apoHDL decay curves for rats were individually modeled using a modified SAAM (simulation, analysis, and modeling) program, Compartmental Analysis Program (Institute of Nuclear Research IPEN, São Paulo, Brazil) (28) as summarized in Figure 1.

Cellular cholesterol efflux. Swiss mouse macrophages were harvested from peritoneal cavity in sterile PBS (0.8% NaCl, 0.006% Na_2HPO_4, 0.02% KCl, and 0.04% KH_2PO_4), with added penicillin (100 U/mL), streptomycin (100 U/mL), and fungizone (2.5 μg/mL) (Gibco BRL, Life Technologies, Rockville, MD). Pelleted cells after centrifugation at 90 × g for 2 min, at 4°C were suspended in the tissue culture medium (RPMI 1640; Gibco BRL, Life Technologies) including 10% FBS (Gibco BRL, Life Technologies), attaining final concentration of

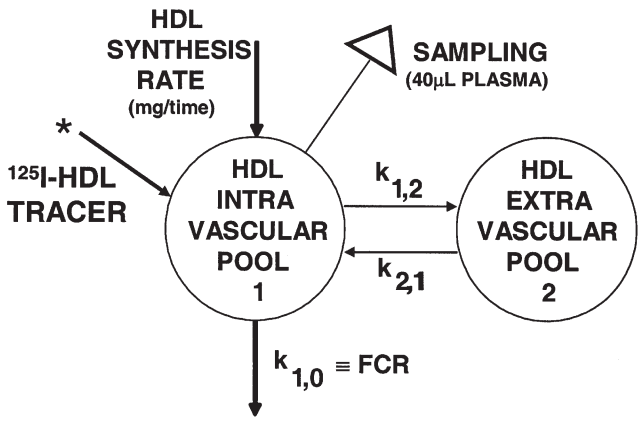

FIG. 1. Schematic model of the HDL kinetics. The model consists of two compartments, or pools, the intravascular and the extravascular compartments. It is assumed that the compartments are in dynamic equilibrium. The $k_{i,j}$ (min^{-1}) values represent the transfer rate of HDL among the compartments. The constants $k_{i,j}$ are associated with the half-life time (min) according to $t_{1/2} = 0.693/k_{i,j}$. In addition, the same quantity of synthesized HDL (mg/time) that is introduced in compartment 1 is removed from the system; thus, synthesis rate of HDL can be estimated using the following formula: HDL synthesis (mg/d) = plasma HDL (mg/mL) × plasma volume (mL) x FCR (h^{-1}) × 24 h/d. This model also assumes that ^{125}I-HDL is injected instantaneously in the intravascular compartment (arrow with asterisk). From compartment 1 a fraction $k_{1,2}$ of HDL is transferred to compartment 2 at each time unit. On the other hand, a fraction $k_{2,1}$ of HDL contained in the extravascular compartment returns to the intravascular compartment in each time unit. The tracer sampling was made in compartment 1 (line with a triangle).

1.5×10^6 cells/well. Cell solution were dispersed into 24-well plastic dishes and kept in a humidified incubator with a 5% CO_2 atmosphere at 37°C, for 24 h. Adherent cells were washed twice in PBS containing antibiotics and 0.1% FA-free albumin (FAFA; Sigma Chemical Co.). Macrophage cholesterol loading was carried out on 24-h incubation in Dulbecco's modified Eagle`s medium (DMEM; Gibco BRL, Life Technologies) containing 0.1% FAFA, 0.5 μCi/mL [4-^{14}C]-free cholesterol ([4-^{14}C]-FC; Dupont-New England Nuclear), and acetylated LDL (50 μg of protein/mL). The purity of the isotope was checked by silica gel TLC and shown to be higher than 98%. Cellular cholesterol pools were equilibrated for 24 h in DMEM/FAFA prior to the incubation with HDL. Cholesterol-enriched cells were next washed twice in PBS-FAFA, and thereafter incubated with NAR and SDR HDL (100 μg of HDL protein/mL) for 6 h. Control incubations were done with DMEM/FAFA only. The medium was drawn into tubes and centrifuged at $125 \times g$ for 10 min to remove cell debris, and the radioactivity was measured in a beta scintillation counter. Cells were rinsed twice with cold PBS/FAFA and twice with cold PBS and extracted with hexane/isopropanol (3:2, vol/vol) (E. Merck). Solvent was evaporated and radioactivity measured. The percentage of ^{14}C-FC efflux was calculated as (^{14}C-FC in the medium/^{14}C-FC in cells plus medium) × 100. The difference between the efflux elicited by HDL plus albumin and that by the albumin-enriched media expresses the HDL-mediated efflux. Protein measurement was done after cell lysis in 0.2 N NaOH (29,30).

Statistical analyses. Data were expressed as mean ± SD. The student's t-test was used for statistical comparison of the data, except in the study of ^{125}I-apoHDL kinetics, where the Mann-Whitney rank sum test was used and data expressed as median and lower quartile. Data evaluation was calculated using GraphPad Prism version 2.01 (GraphPad Software Inc., San Diego, CA). Results of the statistical tests were considered significant at the 95% confidence level ($P < 0.05$).

RESULTS

Similarity of body weight was attained in both experimental groups; however, plasma TAG and TC (Table 1) as well as HDL TAG, TC, PL, and protein concentrations were higher in NAR than in SDR. HDL LPC and PS concentrations were also higher in NAR, but HDL SM, PC, and PE concentrations did not differ between the two groups (Table 2).

We have investigated whether the differences in the HDL composition could be ascribed to differences in post-heparin lipase activity. SDR and NAR HDL particles labeled with L-dipalmitoyl [choline-methyl-^{14}C] were incubated with plasma containing HL according to several combinations possible. In autologous as well as in heterologous incubations HDL-PL hydrolysis *in vitro* was significantly higher when NAR HDL was used as substrate (Table 3). The higher PL hydrolysis of NAR HDL was solely attributed to alteration of the HDL composition and not to differences in intrinsic HL activities between NAR and SDR.

Autologous ^{125}I-apoHDL was infused intra-arterially to further explore whether altered NAR HDL composition brings on modifications in the kinetics of HDL. Total plasma HDL from pools of donor NAR and SDR were labeled with ^{125}I and pulse-infused into the carotid of NAR and SDR rats. Thereafter, radioactivity was measured over the course of 48 h and expressed per mL of plasma. Data derived from the FCR curves expressed as median and lower quartile were, respectively, 0.231 and 1.645 ($n = 10$) in NAR, as compared with 0.140 and 0.109 ($n = 10$) in SDR ($P = 0.012$), indicating a faster rate of HDL metabolism in NAR that can only be explained by a higher HDL synthesis rate in this animal (Fig. 2).

As HDL is the main LP fraction involved in the reverse cholesterol transport system, that is, in the FC removal mechanism from extra-hepatic tissues and its transport to the liver, it was then investigated whether the HDL-mediated cell cholesterol efflux of [^{14}C]-cholesterol enriched macrophages from donor mice was altered in the presence of HDL drawn from NAR. Indeed,

TABLE 1
Characteristics of NAR and SDR Used in This Study

	NAR ($n = 40$)	SDR ($n = 30$)
Body weight (g)	377 ± 26	386 ± 33
Plasma TAG (mg/dL)	116 ± 32[a]	56 ± 18[a]
Plasma TC (mg/dL)	141 ± 30[a]	73 ± 14[a]

Data are expressed as mean ± SD.
[a]$P < 0.0001$, NAR vs. SDR, statistical comparison by student's *t* test.

TABLE 2
HDL Composition in TAG, TC, Protein, Total PL, and Phospholipid Fractions in NAR and in SDR

	NAR (n = 8)	SDR (n = 7)
	mg/dL	
HDL-TAG	3.94 ± 0.78	2.30 ± 0.41[a]
HDL-TC	99 ± 28	55 ± 7[a]
HDL-protein (10)	119 ± 30	37 ± 13[a]
	µmol/mL	
HDL-PL	1.56 ± 0.17	0.78 ± 0.17[a]
HDL-LPC	0.064 ± 0.012	0.023 ± 0.013[a]
HDL-SM	0.035 ± 0.023	0.034 ± 0.028
HDL-PC	0.102 ± 0.047	0.104 ± 0.044
HDL-PS	0.102 ± 0.048	0.044 ± 0.038[b]
HDL-PE	0.029 ± 0.034	0.026 ± 0.033

Data are expressed as mean ± SD. [a]$P < 0.001$, NAR vs. SDR; [b]$P < 0.05$, NAR vs. SDR, statistical comparison by student's t test.

TABLE 3
Post-Heparin Hepatic Lipase Activity on HDL Particles

HDL NAR + HL NAR vs.	HDL SDR + HL SDR	P[a]
92 ± 7 (14)	4.6 ± 2.1(14)	0.0001
HDL NAR + HL SDR vs.	HDL SDR + HL SDR	
89 ± 10 (12)	4.6 ± 2.1 (14)	0.0001
HDL NAR + HL SDR vs.	HDL SDR + HL NAR	
89 ± 10 (12)	3.7 ± 1.4 (9)	0.0001
HDL NAR + HL SDR vs.	HDL NAR + HL NAR	
89 ± 10 (12)	92 ± 7 (14)	0.37
HDL SDR + HL NAR vs.	HDL SDR + HL SDR	
3.7 ± 1.4 (9)	4.6 ± 2.1 (14)	0.25

L-Dipalmitoyl [choline-methyl-^{14}C] HDL particles were incubated with hepatic lipase (HL). HDL particles and HL were drawn from SDR and from NAR, and experiments were designed in a crossover fashion.
Data are expressed as mean ± SD. Values express percent hydrolysis of the radioactive phospholipids; figures in parentheses represent n for the measurement.
[a]Statistical comparison by student's t test

the percent [^{14}C]-cholesterol macrophage efflux was 8.2 ± 2.3 (n = 9) utilizing NAR HDL and 11.2 ± 3.2 (n = 10) utilizing SDR HDL, a difference significant at $P = 0.03$.

DISCUSSION

Albumin plays an important role on the transport of FFA, bilirubin, ions, heavy metals, drugs, hormones, enzymes, and bile acids (31–33). NAR are characterized by analbuminemia, hypercholesterolemia, hypertriacylglycerolemia, and high plasma PL levels (5). These are also characteristics of human analbuminemia (1,2). In addition, several of these features are present in the hypoalbuminemia of NS. The absence of albumin presents potential problems regarding plasma FFA and LPC transport. For instance, LPC and FFA are mainly bound to LP and not to albumin in NAR plasma (7).

The two- to threefold elevated LPC concentration in NAR HDL can be explained by synergistic processes. A faster rate of HDL catabolism in NAR reflects an increased production of HDL, and, consequently, a greater HDL particle number exposure to the action of the HL, which otherwise is normal in these animals. Although Kaysen *et al.* (34) had reported a reduced

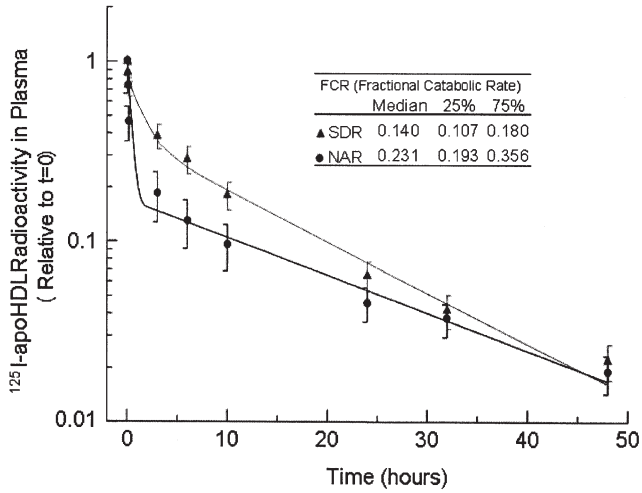

FIG. 2. Plasma radioactivity curves (in 40 µL aliquots) from tail vein blood sequentially drawn over a period of 48 h (3 min, 10 min, 6 h, 10 h, 24 h, 32 h, and 48 h) after an intracarotid autologous infusion of NAR (circles) or of SDR ^{125}I-apoHDL (triangles). Each experimental point represents the mean ± SEM (n = 10) relative to t = 0. Median FCR values (h^{-1}) were derived directly from the kinetic model as the clearance rate from the intravascular pool. NAR FCR 0.231 *vs* SDR FCR 0.140, difference significant at $P = 0.012$. FCR and the HDL-protein concentration in plasma allowed for the calculation of the absolute HDL-protein synthesis rates as mg/d ± SEM, which were 89.8 ± 33.7 mg/d in NAR and 17.4 ± 6.5 mg/d in SDR (difference significant at $P = 0.0122$).

apo-AI fractional catabolic rate in NAR, their apo-AI kinetics had been carried out utilizing apo-AI HDL drawn from normal SDR, and not from NAR. The enhanced conversion of PC to LPC (35) should be accounted for by the HL-induced modification of the LP. In this regard, the HL activity provides the major (~55%) route for PC removal from plasma, which is consistent with the elevated plasma PC concentration observed in humans with HL deficiency (36). The faster plasma clearance of ^{125}I-apoHDL in NAR compared with SDR reflects an increased synthesis rate and plasma HDL concentration simultaneous with an elevated concentration of NAR HDL TAG, PL, and apo-AI. The latter is likely the outcome of the metabolism of TAG-rich particles; a previous study from our laboratory has shown faster plasma TAG and VLDL synthesis rates in NAR than in SDR (10). Our data are compatible with an increased rate of hepatic cholesterol synthesis in NAR as reported by others (37,38).

Hepatic scavenger receptor B type I (SR-BI) protein is not altered in NAR (38). SR-BI internalizes HDL cholesteryl ester but little HDL protein. Thus, the faster turnover rate of HDL protein means that specific receptors, such as kidney cubilin and megalin, must be involved in HDL protein metabolism in NAR (39).

The higher rate of LCAT activity in NAR (7) prevents the concentration of HDL PC from further rising. In other words, an elevated concentration of PC must have occurred early on due to the increased HDL production rate. On the other hand, the higher HDL PS concentration in NAR may have rendered the HDL particle more susceptible to the action of HL, as previously reported

(40). Furthermore, greater plasma apoE and apo-AI concentrations in NAR, attributed mostly to the apoE-loaded HDL (7), may also activate the HDL PC hydrolysis by HL (7,35,41–45). Finally, LPC is known to modulate the metabolism of lipoproteins upon modifying their clearance *in vivo* and *in vitro* (46,47).

Considering that HDL is a critical protecting factor against premature atherosclerosis (48,49), we investigated the role of this particle on the removal of unesterified cholesterol from mouse peritoneal macrophages. This is the first step in the reverse cholesterol transport system (RCT) that ultimately delivers peripheral cholesterol to the liver (43,50). As compared with SDR the NAR HDL capacity for FC efflux from the lipid-loaded macrophages is significantly impaired. In this regard, it is known that the PL content and composition are important factors determining the HDL capacity for FC efflux from peripheral cell plasma membranes (51–53). For instance, HDL PC hydrolysis by phospholipase A2 slows down the rate of the SR-BI-mediated cholesterol efflux (53). Thus, the increased LPC content in NAR HDL may have contributed simultaneously to the impaired cell FC efflux rate and to the faster hepatic HDL uptake. Increased formation of HDL LPC and decreased HDL-dependent FC efflux from lipid-loaded mouse macrophages have also been reported in the human HDL pretreated with human secretory phospholipase (54). However, an impaired adenosine triphosphate-binding cassette transporter A1 receptor expression in NAR could not be ruled out and needs further investigation.

In summary, this study indicates that in NAR, plasma concentrations of TAG, PL, and TC are elevated largely due to the HDL fraction reflecting higher rate of HDL protein synthesis. The increased HDL lipid content renders the particle more susceptible to the otherwise unaltered intrinsic HL activity. Moreover, the efficiency of the NAR HDL to remove cell cholesterol is impaired. Therefore, alterations in the HDL metabolic profile described in NAR help explain disturbances of the RCT.

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Effects of *cis*-9,*trans*-11 CLA in Rats at Intake Levels Reported for Breast-fed Infants

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ABSTRACT: CLA intake in exclusively breast-fed infants is close to levels found to have physiological effects in animals. However, in the majority of studies mixtures of CLA isomers have been used and the independent effects of the major CLA isomer in human milk, *cis*-9,*trans*-11 CLA, at the intake level in exclusively breast-fed infants have hardly been studied. We therefore studied the effects of *cis*-9,*trans*-11 CLA on plasma lipids and glucose, immune function, and bone metabolism in growing rats. Thirty male Sprague-Dawley rats (n = 10/group) were fed either 20 mg/kg/d *cis*-9,*trans*-11 CLA and 20 mg/kg/d sunflower oil (CLA20), 40 mg/kg/d *cis*-9,*trans*-11 CLA (CLA40), or 40 mg/kg/d sunflower oil (placebo) for 8 wk. No significant differences between groups were found in plasma lipids, glucose, insulin, C-reactive protein, or lipid peroxidation. Liver fat content was lowest in the CLA20 group. *In vitro* interleukin 2 (IL-2) production increased, and tumor necrosis factor alpha, IL-1ß, prostaglandin E_2, and leukotriene B_4 production decreased in the CLA20 group. No differences between groups were detected in IL-4, IL-6, or interferon gamma production, plasma osteocalcin, insulin-like growth factor, or urinary deoxypyridinoline crosslinks. Plasma tartrate-resistant acid phosphatase 5b activity was significantly increased in the CLA40 group. The results indicate anti-inflammatory effects and enhanced T-cell function for the CLA20 group. No adverse effects were seen in the CLA20 group, whereas indications of increased bone resorption rate were observed in the CLA40 group.

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CLA, a group of positional and geometric isomers of linoleic acid (*cis*-9,*cis*-12 18:2), has been shown to possess a multitude of beneficial physiological effects, including anticarcinogenic, antiatherogenic, and antidiabetic properties as well as effects on body composition (1,2). Evidence also suggests that it may enhance selective immune functions, while ameliorating negative effects of abnormal immunological responses, possibly via altered cytokine and immunoglobulin production (3). Effects of CLA on bone metabolism have been reported in few studies, with both increased (4) and decreased (5) bone formation rates being reported.

However, adverse effects in both animals and humans have also been reported. Increased liver (6,7) and spleen weight (6) and/or increase in liver lipids (up to 5-fold) (7,8) have been found in mice fed a CLA mixture or *trans*-10,*cis*-12 CLA. Indications of increased insulin resistance were observed in mice fed a CLA mixture (6) and in overweight subjects fed *trans*-10,*cis*-12 CLA (9). Serum C-reactive protein (CRP), an acute-phase reactant, was doubled in subjects receiving *trans*-10,*cis*-12 CLA (10). Two studies have shown a several-fold increase in lipid peroxidation, measured as urinary 8-iso-prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in subjects fed a CLA mixture or *trans*-10,*cis*-12 CLA (10,11). Evidence from studies using pure isomers thus suggests that the isomer responsible for the adverse effects may be *trans*-10,*cis*-12 CLA. However, in a recent study *cis*-9,*trans*-11 CLA decreased insulin sensitivity by 15% and increased lipid peroxidation by 50% in abdominally obese men (12).

CLA intake relative to body weight is significantly higher in exclusively breast-fed infants than in adults, and is close to levels reported to have beneficial physiological effects in animals (~23 mg/kg body wt/d) (13,14). Human milk CLA concentrations have been reported to range from 2.23 to 5.43 mg/g fat (15). Milkfat-based infant formulas also contain CLA, whereas it is generally not detected in vegetable oil–based formulas (15). Data from both animal and human studies suggests that CLA intake during critical phases of development may have effects later in life (16–18). Most earlier studies have used CLA isomer mixtures containing equal amounts of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers. The independent effects of the major CLA isomer in human milk, *cis*-9,*trans*-11 CLA, at intake levels corresponding to those in exclusively breast-fed infants have not been studied. We therefore studied the physiological effects of *cis*-9,*trans*-11 CLA in a developing animal model.

EXPERIMENTAL PROCEDURES

The study was conducted at the Laboratory Animal Centre of the University of Helsinki, Finland. The protocol was approved by the Laboratory Animal Ethics Committee of the University of Helsinki. Three-week-old male Sprague-Dawley rats (n = 30)

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Abbreviations: Con A, concavalin A; CRP, C-reactive protein; DPD, deoxypyridinoline; IFN-γ, interferon gamma; IGF-1, insulin-like growth factor; IL, interleukin; LPS, lipopolysaccharide; LT, leukotriene; PG, prostaglandin; TNF-α, tumor necrosis alpha; TRACP, tartrate-resistant acid phosphatase.

were purchased from Harlan (Horst, the Netherlands). The animals were housed in plastic cages, two rats per cage, in a temperature- and humidity-controlled animal facility, with 12-h light/dark cycle. After a 4-d adaptation period, they had free access to standard rodent laboratory chow (Teklad Global 18% Protein Rodent Diet, Harlan, Horst, the Netherlands) for the 8-wk feeding period. The chow contained 5.7 g fat/100 g, of which 17.1% was saturated FA (SFA), 22.6% monounsaturated FA (MUFA), and 60.2% PUFA. Commercial soy-based infant formula (SoijaTutteli, Valio, Helsinki, Finland) was provided as drinking fluid. Infant formula was used to simulate the diet of infants, with the formula being the main source of energy and nutrients. The infant formula contained 1.9 g protein, 7.0 g carbohydrates, and 3.5 g fat per 100 mL formula. Of the fat, 32.8% was SFA, 50.7% MUFA, 13.4% n-6 PUFA, and 2.7% n-3 PUFA. Traces of CLA were detected.

The animals were randomly assigned to three groups, and received 40 mg/kg/d sunflower oil (placebo), 20 mg/kg/d *cis*-9,*trans*-11 CLA and 20 mg/kg/d sunflower oil (CLA20), or 40 mg/kg/d *cis*-9,*trans*-11 CLA (CLA40). CLA intake in the CLA20 group was formulated to simulate intake in exclusively breast-fed infants (15). *cis*-9,*trans*-11 CLA as TAG was purchased from Natural Inc. (Hovdebygda, Norway) and contained 91.1% *cis*-9,*trans*-11 CLA, 4.8% oleic acid, 2.4% *trans*-10, *cis*-12 CLA, and 1.3% other conjugated 18:2 isomers. The concentration of other FA was <0.1%. Both CLA and a placebo were orally administered daily by the same person throughout the study. Body weight was recorded weekly, and doses of CLA and placebo were adjusted according to weight gain. The initial daily dose was 2 mg and was increased according to weight gain up to 6 mg/d in the CLA20 group and 12 mg/d in the CLA40 group during the last week to provide daily doses of 20 and 40 mg/kg.

Sampling. Mid-study (4 wk), nonfasting blood samples were obtained from the lateral tail vein, centrifuged, and stored at –70°C. A metabolic cage was used to collect urine samples prior to sacrifice. Urine was aliquoted and stored at –70°C. The animals were without food for 16 h, and were killed by CO_2 asphyxiation. After sacrifice selected organs (liver, spleen, pancreas, heart, brain) were removed and weighed, and blood was drawn from the caudal vena cava into sodium heparin tubes. Blood for lymphocyte stimulations was stored at 4°C. The rest of the blood was aliquoted, snap frozen in liquid nitrogen, and stored at –70°C.

Samples of liver and heart were taken from all rats ($n = 30$) for the analysis of FA composition, snap frozen in liquid nitrogen, and stored at –70°C. Samples of liver, heart, and pancreas for histological evaluation were placed in 10% buffered formalin for fixation and storage.

Glucose and lipid metabolism analyses. Plasma glucose, TG, total cholesterol, HDL, and LDL were analyzed with an automated blood analyzer (Konelab, Thermo Clinical Instruments, Vantaa, Finland). Plasma insulin and CRP were determined using EIA kits from SPI-BIO (Massy Cedex, France) and Alpha Diagnostic International (San Antonio, TX), respectively.

Plasma and tissue FA analyses. Fat was extracted from 200 μL plasma with dichloromethane-methanol (2:1) (19) and from 50–100 mg liver or heart tissue with hexane-isopropanol (3:2) (20). Total FA were methylated with acidic methanol (5% H_2SO_4) (21). Liver FAME concentration was determined by adding internal standard (19:0 methyl ester) to the extraction solution, and the result was expressed as μg per mg weighed liver tissue from duplicate tissue samples. Half of the ventricular part of the heart was taken for homogenization and extraction. The percentage composition of plasma and tissue methylated total FA from 14:0 to 22:6n-3 was determined by a HP 6890 GC (Hewlett Packard, Palo Alto, CA) and Chemstation (version A.06.03) with a 25-m NB-351 column (i.d. 0.32 mm, phase layer 0.20 mm, HNU-Nordion Ltd Oy, Helsinki, Finland), split injection, with hydrogen as carrier gas. A temperature program from 160°C to 230°C was used. The percentage composition of FAME was normalized to 100%.

Lipid peroxidation. Urine samples collected prior to sacrifice were analyzed for 8-iso-$PGF_{2\alpha}$, an indicator of nonenzymatic lipid peroxidation. Nonextracted urine was analyzed using highly specific and sensitive radioimmunoassay as previously described (22). Concentrations of 8-iso-$PGF_{2\alpha}$ were adjusted for creatinine values measured with a commercial kit (IL test, Monarch Instrument, Amherst, NH).

Histological analyses. Formalin-fixed samples were dehydrated and embedded in paraffin wax using standard histological techniques. The tissues were sectioned to the thickness of 5 μm, and the slices were mounted on glass slides and stained with hematoxylin and eosin. The slides were examined using a light microscope by a pathologist (J.L.) blinded to grouping, with emphasis on vacuolation (fat accumulation) and inflammation. The tissues for histological examination and pathological endpoints were selected based on findings in previous studies (6,23). Inflammatory changes in the liver and hepatocellular vacuolation were graded from 1 to 5, with 1 depicting incidental or minimal changes and 5 depicting severe or extensive changes. Besides grading, the nature of the alterations, including main inflammatory cell types and type of vacuolation, as well as their extent (focal to diffuse) were recorded.

Blood lymphocyte stimulation. Lymphocytes were isolated from 2 mL heparin blood by Ficoll-Paque centrifugation (24). Blood was diluted with 10% FCS/RPMI culture media (1:1). Cells were counted in a Neubauer Improved counting chamber and diluted in the culture media to achieve a concentration of 2×10^6/mL. Cells were then frozen at –70°C.

Lymphocytes were stimulated using concavalin A (Con A; Amersham Pharmacia Biotech, Uppsala, Sweden), a T-cell mitogen, and lipopolysaccharide (LPS; Sigma Chemicals, St. Louis, MO), a B-cell mitogen. Mitogens were diluted using 10% FCS/RPMI media. Final concentrations of Con A and LPS were 5 mg/L and 10 mg/L, respectively. Mitogen concentrations and incubation times were selected based on earlier studies.

Diluted mitogens were pipeted into 96-well microplates and cell suspensions (2×10^6/mL) were added to each well containing mitogen or blank (10% FCS/RPMI). Each sample was ana-

lyzed in triplicate. The plates were incubated at 37°C, 5% CO_2 for 4 and 24 h. Samples were centrifuged, and supernatants were collected. Supernatants of replicate wells were pooled and stored at –70°C until analyzed. Two time points were used in order to detect early events and possible differences in cytokine profiles and responses at different time points.

In vitro production of cytokines. All cytokines were analyzed using ELISA kits from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. TNF-α, IL-6, IL-1β, and PGE_2 were measured in the supernatant of LPS-stimulated cells. IL-2, IL-4, IFN-γ, and LTB_4 were analyzed in supernatants of Con A–stimulated cells. All samples were analyzed in duplicate. Control samples included in each kit were analyzed with each batch of samples.

Markers of bone metabolism. Plasma insulin-like growth factor (IGF-1) and osteocalcin were analyzed as markers of bone formation. Urinary deoxypyridinoline (DPD) crosslinks and plasma tartrate-resistant acid phosphatase (TRACP) 5b, derived from osteoclasts, were analyzed as markers of bone resorption. ELISA/EIA kits were used to analyze plasma IGF-1 (IDS Ltd, Boldon, UK), plasma osteocalcin (BTI Inc, Stoughton, MA), and urinary DPD (Metra Total DPD, Quidel Corp, San Diego, CA). DPD concentrations were adjusted for creatinine, analyzed with a commercial kit (IL test; Monarch Instrument, Amherst, NH). A solid-phase immunofixed-enzyme activity assay (SBA Sciences, Turku, Finland) was used to measure TRACP 5b in plasma. This assay is specific for active TRACP 5b molecules freshly liberated into the circulation from osteoclasts. Control samples included in each kit were analyzed with each batch of samples.

Statistical analyses. Values are means ± SD. Statistical analyses were performed by using SPSS (version 10.0, SPSS Inc, Chicago, IL). One-way ANOVA was used to detect differences between groups. Results for graded histological analysis for microvesicular hepatocellular vacuolation were analyzed using the Kruskall Wallis test. Spearman correlation coefficient was determined from pairwise correlations. A $P < 0.05$ was considered significant.

RESULTS

Growth. Growth was similar in the three groups (data not shown). Initial average weights were 84 ± 13 g, 87 ± 13 g, and 89 ± 14 g, and final average weights were 318 ± 26 g, 332 ± 18 g, and 329 ± 34 g in the CLA20, CLA40, and control groups, respectively. Infant formula was the main source of energy and provided on average 70% of total energy intake (as measured by milk and chow consumption).

No differences between groups were observed in the weights of the heart (1.2 ± 0.1 g, 1.2 ± 0.1 g, and 1.2 ± 0.1 g in the CLA20, CLA40, and control groups, respectively), liver (9.8 ± 1.3 g, 9.8 ± 0.9 g, and 10.3 ± 1.4 g in the CLA20, CLA40, and control groups, respectively), spleen (0.7 ± 0.1 g, 0.7 ± 0.1 g, and 0.7 ± 0.1 g in the CLA20, CLA40, and control groups, respectively), pancreas (1.2 ± 0.3 g, 1.4 ± 0.3g, and 1.4 ± 0.2 g in the CLA20, CLA40, and control groups, respectively), or brain (1.8 ± 0.1g, 1.9 ± 0.0g, and 1.8 ± 0.1g in the CLA20, CLA40, and control groups, respectively). However, liver fat content in the CLA20 group was significantly lower (1.62%) than in the control (1.81%) or CLA40 groups (1.88%) ($P = 0.02$).

Plasma and tissue FA. *cis*-9,*trans*-11 CLA in plasma and liver showed a dose-response increase in the three groups (Table 1), but not in heart tissue (data not shown). Its proportion in heart tissue (0.02%, 0.05%, and 0.05% in the control, CLA20, and CLA40 groups, respectively) was 2- to 3-fold lower than in plasma and liver. No other differences between groups were observed in heart FA compositions. In the liver, stearic acid (18:0) was significantly lower in the CLA40 group than in the CLA20 group, as a percentage of total FA ($P = 0.04$). Linoleic acid (18:2n-6; LA) was significantly lower in the CLA20 group than in the CLA40 group both in the liver (as % total FA) ($P = 0.02$) and plasma ($P = 0.02$), but did not differ from controls. The proportion of DHA (22:6n-3) in plasma was higher in both CLA groups than in controls ($P = 0.03$), whereas in the liver, DHA (as % total FA) was lowest in the CLA40 group ($P = 0.004$). Δ5 (20:4n-6/20:3n-6), Δ6 [(18:3n-6 + 20:3n-6)/18:2n-6], and Δ9 (18:1/18:0) desaturation indices did not differ between groups in plasma or in the liver.

Lipid and glucose metabolism. Plasma total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride concentrations were similar in all three groups (Table 2).

Nonfasting plasma glucose at 4 wk and fasting plasma glucose at 8 wk tended to increase with increasing CLA concentrations, whereas plasma insulin and CRP tended to decrease, but the differences between groups were not significant (Table 2).

After adjusting for creatinine, no differences between groups in 8-iso-$PGF_{2\alpha}$ were observed (Table 2). 8-iso-$PGF_{2\alpha}$ concentrations were not correlated with CRP, glucose, or insulin.

Histological analyses. Histological analyses of the heart, liver, and pancreas revealed incidental focal inflammatory changes in the liver of three rats (one in the control group, two in the CLA40 group). The foci consisted mostly of macrophages, lymphocytes, and plasma cells, but cellular debris and a few other leucocytes were sometimes present. There was no predisposition toward any of the acinar zones, and the foci were no more than 5–10 hepatocytes in diameter. These types of changes are commonly detected in various strains of normal rats; their etiology is uncertain but they are of no functional significance. Patchy or diffuse hepatocellular microvesicular vacuolation was observed in all groups (data not shown). Marked (grade 4) hepatocellular vacuolation was observed in four rats, all from the CLA40 group. The difference in graded changes between groups (2.4, 2.5, and 3.1 for the control, CLA20, and CLA40 groups, respectively) was, however, not significant.

Cytokines. No differences between groups were seen in IL-2 at 4 h, whereas at 24 h concentrations in the CLA20 group were significantly higher compared to controls (207.8 pg/mL vs. 93.9 pg/mL, $P < 0.01$) (Table 3). The CLA40 group did not differ from controls. Concentrations increased 4-fold between 4 h and 24 h in the CLA20 group, but only doubled in the two other groups.

TABLE 1
Selected Plasma and Liver FA in Rats Fed Control Diet, 20 mg/kg CLA, or 40 mg/kg CLA[a]

	Control	CLA20	CLA40
Plasma (% total FA)			
16:0	19.47 ± 0.91	18.98 ± 0.72	19.20 ± 1.33
18:0	11.86 ± 1.17	11.73 ± 1.10	11.08 ± 0.68
total SFA	31.75 ± 1.52	31.11 ± 1.16	30.67 ± 1.46
18:1	11.30 ± 0.87	11.08 ± 1.37	11.80 ± 1.49
total MUFA	13.78 ± 1.08	13.49 ± 1.50	14.29 ± 1.64
18:2n-6	17.07 ± 1.23^{ab}	16.48 ± 1.12^{a}	18.13 ± 0.98^{b}
cis-9,*trans*-11 CLA	0.01 ± 0.02^{a}	0.11 ± 0.04^{b}	0.16 ± 0.06^{c}
18:3n-3	0.34 ± 0.06^{ab}	0.29 ± 0.06^{a}	0.37 ± 0.08^{b}
20:4n-6	33.29 ± 2.22	34.22 ± 2.37	32.28 ± 2.92
20:5n-3	0.22 ± 0.09	0.26 ± 0.13	0.27 ± 0.04
22:6n-3	2.56 ± 0.45^{a}	3.02 ± 0.43^{b}	2.78 ± 0.31^{ab}
total PUFA	54.46 ± 1.89	55.39 ± 1.57	55.03 ± 2.68
18:1/18:0	0.96 ± 0.16	0.96 ± 0.21	1.07 ± 0.16
20:4n-6/20:3n-6	120.7 ± 20.9	114.4 ±21.4	106.8 ± 23.0
(18:3n-6 + 20:3n-6)/18:2n-6	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
Liver (% total FA)			
16:0	20.93 ± 0.76	21.02 ± 0.66	21.42 ± 0.62
18:0	14.34 ± 1.64^{ab}	14.44 ± 1.18^{a}	13.40 ± 0.93^{b}
total SFA	35.75 ± 1.26	35.92 ± 1.12	35.32 ± 0.74
18:1	14.48 ± 2.03	15.13 ± 1.72	15.95 ± 1.75
total MUFA	17.40 ± 2.09	17.90 ± 1.79	18.81 ± 1.82
18:2n-6	19.64 ± 2.36^{ab}	18.63 ± 1.52^{a}	20.22 ± 1.35^{b}
cis-9,*trans*-11 CLA	0.03 ± 0.01^{a}	0.11 ± 0.02^{b}	0.16 ± 0.02^{c}
18:3n-3	0.67 ± 0.17	0.60 ± 0.11	0.68 ± 0.08
20:4n-6	19.89 ± 2.29	19.86 ± 1.64	18.58 ± 1.39
20:5n-3	0.28 ± 0.05	0.25 ± 0.04	0.28 ± 0.04
22:6n-3	5.20 ± 0.84^{ab}	5.49 ± 0.43^{a}	4.73 ± 0.58^{b}
total PUFA	46.89 ± 1.13	46.18 ± 1.38	45.87 ± 1.45
18:1/18:0	1.04 ± 0.26	1.06 ± 0.20	1.21 ± 0.20
20:4n-6/20:3n-6	68.84 ± 9.66	61.67 ± 6.71	59.63 ± 11.24
(18:3n-6 + 20:3n-6)/18:2n-6	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
Liver (μg/mg tissue)			
16:0	3.80 ± 0.66	3.60 ± 0.46	3.94 ± 0.71
18:0	2.56 ± 0.26	2.39 ± 0.25	2.26 ± 0.22
total SFA	6.45 ± 0.86	5.99 ± 0.67	6.51 ± 0.94
18:1	2.65 ± 0.70	2.46 ± 0.50	2.89 ± 0.84
total MUFA	3.18 ± 0.80	2.98 ± 0.66	3.64 ± 0.94
18:2n-6	3.59 ± 0.89	3.03 ± 0.61^{a}	3.82 ± 0.75^{b}
cis-9,*trans*-11 CLA	0.00 ± 0.00^{a}	0.02 ± 0.00^{b}	0.03 ± 0.01^{c}
18:3n-3	0.12 ± 0.05	0.11 ± 0.03	0.13 ± 0.03
20:4n-6	3.56 ± 0.42	3.29 ± 0.32	3.41 ± 0.33
20:5n-3	0.05 ± 0.02	0.04 ± 0.01	0.05 ± 0.02
22:6n-3	0.93 ± 0.13	0.88 ± 0.11	0.87 ± 0.09
total PUFA	8.49 ± 1.27	7.66 ± 1.00	8.54 ± 1.12

[a]Values are mean ± SD, $n = 10$. Control diet, 40 mg/kg/d sunflower oil; CLA20, 20 mg/kg/d *cis*-9,*trans*-11 CLA; CLA40, 40 mg/kg/d *cis*-9,*trans*-11 CLA. Values in a row not sharing a common superscript differ from each other ($P < 0.05$, ANOVA).

The concentrations of IL-4, IL-6, and IFN-γ did not differ between groups at 4 h or 24 h (Table 3). IL-4 concentrations decreased from 4 h to 24 h, but were low at both time points in all groups. Concentrations of IL-6 increased 1.4- to 3.2–fold, and those of IFN-γ doubled between 4 h and 24 h in all groups (Table 3).

TNF-α concentrations were significantly lower at 4 h in the CLA20 group (56.0 pg/mL) than in the control or CLA40 group (138.2 and 137.8 pg/mL, respectively; $P < 0.001$). The same difference was seen also at 24 h (42.7 pg/mL, 130.6 pg/mL, and 112.8 pg/mL for the CLA20, CLA40, and control groups, respectively; $P < 0.01$). The control and CLA40 groups did not differ at either time point (Table 3).

IL-1β was significantly lower in the CLA20 group at 24 h than in the two other groups ($P = 0.01$). A similar tendency was seen also at 4 h, but the differences were not significant. Concentrations increased 3-fold from 4 h to 24 h in all groups.

PGE_2 and LTB_4 concentrations were significantly lower in the CLA20 group than in the two other groups at 4 h ($P < 0.05$), but not at 24 h (Table 3). PGE_2 concentrations increased 8- to

TABLE 2
Lipoproteins and Selected Other Blood Parameters in Rats Fed Control Diet, 20 mg/kg CLA or 40 mg/kg CLA[a]

	Control	CLA20	CLA40
CRP (ng/mL)	343.8 ± 61.1	330.2 ± 45.0	299.0 ± 33.0
Glucose (mmol/L) 4 wk[b]	9.0 ± 2.1	8.4 ± 1.4	10.3 ± 2.9
8 wk[c]	5.9 ± 0.3	6.2 ± 0.4	6.2 ± 0.4
Insulin (ng/mL)	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
Cholesterol (mmol/L)	2.3 ± 0.2	2.3 ± 0.5	2.6 ± 0.7
LDL cholesterol (mmol/L)	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
HDL cholesterol (mmol/L)	1.9 ± 0.2	1.9 ± 0.5	1.9 ± 0.4
Triacylglycerols (mmol/L)	0.5 ± 0.1	0.5 ± 0.2	0.7 ± 0.4
8-iso-$PGF_{2\alpha}$ (ng/mmol creatinine)	107.9 ± 31.5	113.2 ± 22.7	130.3 ± 40.9
IGF-1 (ng/mL)	852 ± 348	942 ± 176	1033 ± 129
Osteocalcin (ng/mL)	183 ± 33	206 ± 22	182 ± 30
Urinary DPD (nmol/mmol crea)	253 ± 50	241 ± 79	215 ± 29

[a]Values are mean ± SD, $n = 10$. Control diet, 40 mg/kg/d sunflower oil; CLA20, 20 mg/kg/d cis-9,*trans*-11 CLA and 20 mg/kg/d sunflower oil; CLA40, 40 mg/kg/d *cis*-9,*trans*-11 CLA.
[b]Nonfasting plasma glucose concentrations.
[c]Fasting plasma glucose concentrations.

16-fold from 4 h to 24 h, while those of LTB_4 remained unchanged in the control and CLA40 groups, but increased 2.5-fold in the CLA20 group.

TNF-α was significantly correlated with IL-1β ($r = 0.695$, $P = 0.006$), IL-6 ($r = 0.698$, $P = 0.005$), PGE_2 ($r = 0.611$, $P = 0.016$), and LTB_4 ($r = 0.776$, $P = 0.003$), and negatively correlated with IL-2 ($r = -0.577$, $P = 0.024$). Significant correlations were also seen between IL-1β and IL-6 ($r = 0.630$, $P = 0.016$), and between PGE_2 and LTB_4 ($r = 0.841$, $P = 0.001$).

Bone metabolism. Plasma IGF-1 showed a tendency to increase with increasing CLA intake, but the differences between groups were not significant (Table 2). Plasma osteocalcin concentrations were also similar in all groups (Table 2). In the CLA40 group, plasma TRACP 5b activity was 40% higher than in the control and CLA20 groups ($P < 0.05$; Fig. 1). However, no differences between groups were seen in the other marker of bone resorption, urinary DPD (Table 2). TRACP 5b activity was correlated with plasma PGE_2 concentrations ($r = 0.602$, $P = 0.018$)

DISCUSSION

The effects of 20 and 40 mg/kg/d *cis*-9,*trans*-11 CLA in growing rats were studied. Minor differences between groups in plasma and liver FA were observed, but we did not find indications of altered LA or linolenic acid metabolism. A recent human study reported an increase in plasma LA and a decrease in Δ6-desaturation by both 3 g/d *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA (25), whereas, in line with our study, another study found no effects with 0.59–2.38 g/d of the same isomers (26).

Microvesicular steatosis of the liver was found in all groups in the present study. This was probably due to all diets containing more fat than the general rodent diet. Liver fat content in the CLA20 group was significantly lower than in the two other groups. The inflammatory changes seen in the liver of three rats were considered unrelated to the treatments, because they were present both in the control and CLA40 groups and are commonly detected in various strains of normal rats. Increased liver weight (6,12) and hepatocellular hypertrophy (6,23) have been

TABLE 3
In vitro Cytokine Production by Stimulated Lymphocytes in Rats Fed 20 mg/kg CLA, 40 mg/kg CLA, or Control Diet[a]

	4 h			24 h		
	Control	CLA20	CLA40	Control	CLA20	CLA40
IL-2	49.3 ± 1.2	51.0 ± 2.15	50.5 ± 1.21	93.9 ± 43.7[b]	207.8 ± 52.9[a]	138.0 ± 97.4[b]
IL-4	49.9 ± 0.8	48.8 ± 1.7	49.6 ± 1.2	26.7 ± 1.2	26.7 ± 1.5	27.7 ± 1.6
IL-6	87.7 ± 6.1	134.4 ± 146.7	114.7 ± 67.9	285.0 ± 65.8	275.2 ± 42.6	271.9 ± 110.0
TNFα	137.8 ± 34.5[b]	56.0 ± 16.3[a]	138.2 ± 30.9[b]	112.8 ± 42.6[b]	42.7 ± 55.4[a]	130.6 ± 42.7[b]
IL-1β	12.0 ± 5.1[ab]	9.8 ± 2.9[a]	15.7 ± 4.5[b]	41.6 ± 13.0[b]	26.9 ± 3.8[a]	49.9 ± 7.6[b]
IFN-γ	23.0 ± 5.26	27.0 ± 13.1	26.3 ± 3.4	48.9 ± 14.5	46.7 ± 18.1	51.8 ± 15.8
PGE_2	107.4 ± 65.1[b]	61.3 ± 19.7[a]	136.5 ± 42.6[b]	1,016.7 ± 385.2	1,016.2 ± 326.5	1,088.6 ± 764.2
LTB_4	55.5 ± 14.1[b]	17.0 ± 3.3[a]	52.1 ± 19.0[b]	50.4 ± 16.6	43.8 ± 13.0	57.9 ± 19.3

[a]Values are mean ± SD, all values are in pg/mL, $n = 10$. Control diet, 40 mg/kg/d sunflower oil; CLA20, 20 mg/kg/d *cis*-9,*trans*-11 CLA and 20 mg/kg/d sunflower oil; CLA40, 40 mg/kg/d *cis*-9,*trans*-11 CLA. Values in a row not sharing a common superscript differ from each other ($P < 0.05$, ANOVA).

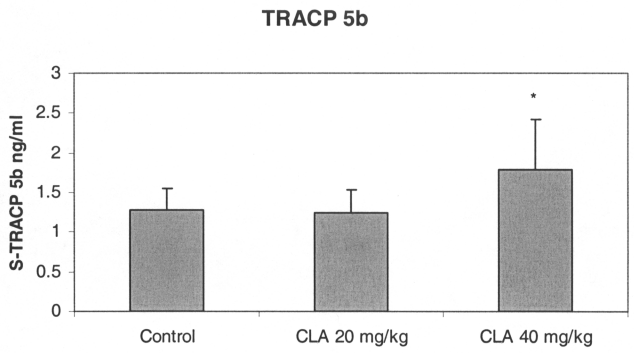

FIG. 1. Plasma tartrate-resistant acid phosphatase (TRACP) 5b concentrations in rats fed control diet, 20 mg/kg/d CLA, or 40 mg/kg/d CLA. An asterisk (*) indicates that the result is significantly different from the two other groups ($P = 0.015$, ANOVA).

reported following CLA feeding in rats and mice. Liver fat content increased in mice fed diets containing 0.5–1.5% (wt/wt) CLA mixture (8) or 0.5% *trans*-10,*cis*-12 CLA (7), but not when the diet contained 0.5% *cis*-9,*trans*-11 CLA (7). In an oral toxicity study, hepatocellular vacuolation was observed in both controls and rats fed 1, 5, or 15 wt% CLA mixture containing equal amounts of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers, but there was no evidence of fat accumulation in the liver (23). Also our results do not suggest fat accumulation at the intake levels used in the present study.

Most previous animal and human studies do not suggest a significant effect for *cis*-9,*trans*-11 CLA on plasma lipids or glucose (27–32), thereby supporting our results. Increased insulin concentrations have been observed in several studies in mice after 1 wt% CLA supplementation (6,33,34), whereas in Zucker prediabetic fatty rats 1.5% CLA mixture (*cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA 50:50) improved insulin action and hyperinsulinemia (35,36). In type 2 diabetic subjects, CLA supplementation (3 g/d *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA, 1:1) increased fasting plasma glucose and reduced insulin sensitivity (37), whereas no effect of isomer on plasma insulin was detected in healthy males fed different doses (0.59–2.38 g/d) of *cis*-9,*trans*-11 CLA or *trans*-10,*cis*-12 CLA (38).

A proinflammatory and pro-oxidative effect for CLA has been suggested by human studies showing increased CRP and lipid peroxidation in subjects fed 3 g/d *trans*-10,*cis*-12 CLA (10), 3 g/d *cis*-9,*trans*-11 CLA (12) or 4.2 g/d CLA mixture (11,39) and by studies showing increased IL-6 secretion in mice fed pure 0.5 wt% *cis*-9,*trans*-11 (40,41) or *trans*-10,*cis*-12 CLA (40). Two recent studies did not, however, find any effects on CRP in patients with type 2 diabetes fed 3 g/d CLA mixture (37) or in healthy males fed *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA 0.59–2.38 g/d (42). In addition, decreased antigen-induced IL-6 production in rats (43) and pigs (44) has also been reported. We also found no evidence of increased CRP or IL-6 with 20 or 40 mg/kg *cis*-9,*trans*-11 CLA. No differences between groups in urinary isoprostanes were detected after adjusting for creatinine, although the higher CLA dose in the present study, 40 mg/kg/d, is comparable to the dose used by Riserus *et al.* (12). Thus, our results do not suggest proinflammatory or pro-oxidative effects for *cis*-9,*trans*-11 CLA at 20 or 40 mg/kg daily doses.

Anti-inflammatory effects, on the other hand, were observed in the present study as production of the proinflammatory cytokines TNF-α and IL-1β decreased in the CLA20 group. Why the effect was observed only in the CLA20 but not the CLA40 group is not obvious. TNF-α and IL-1β decreased also in healthy subjects fed 3 g/d CLA mixture (45) and basal TNF-α secretion of peritoneal macrophages decreased in rats fed 1 wt% CLA (43). A recent study in weaned pigs showed an overall anti-inflammatory effect: 2 wt% CLA prevented an increase in mRNA expression and production of TNF-α and IL-6 in spleen and thymus, whereas the expression of anti-inflammatory IL-10 was enhanced (44). This and most other studies (41,46,47) have found no effects on IFN-γ.

IL-2 plays a central role in cell-mediated immune response. Consistent with our results, several previous studies have reported increased IL-2 concentrations in response to CLA, suggesting an improvement of the proliferative abilities of lymphocytes. Studies in rats and mice using CLA mixture 0.3–1 wt% of diet have shown increased *in vitro* IL-2 production (48,49,50). The only other study using purified isomers found, however, no effects for 0.5 wt% *cis*-9,*trans*-11 CLA or *trans*-10,*cis*-12 CLA on splenocyte IL-2 production in mice (40). In pigs infected with type-2 porcine circovirus, IL-2 mRNA expression was upregulated in the group fed CLA and was associated with less severe clinical manifestation of the disease (51). Based on the functions of IL-2, this data implies an important role for CLA in immune development.

IL-4 and PGE_2 play a role in IgE-mediated hypersensitivity reactions. Two studies in mice fed CLA mixture (52) or purified *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers (40) reported decreased IL-4 secretion in stimulated lymphocytes. In our study, IL-4 concentrations were low at both time points in all groups, including the control group, and no effect for CLA was observed. Reduced plasma PGE_2 levels were associated with decreases in splenic and exudate cell LTB_4 production in rats (48), which was seen as an indication of the potential of CLA to attenuate allergic reactions. Also in the present study, *in vitro* production of both PGE_2 and LTB_4 decreased and a highly significant correlation between the two was observed. In a guinea pig model of hypersensitivity, ovalbumin-induced release of LTB_4 in lungs, trachea, and bladder was not affected by CLA feeding, but the production of LTC_4, LTD_4, and LTE_4 significantly decreased in lungs (53). In the same guinea pig model, CLA significantly reduced PGE_2 secretion by sensitized tracheae (54). 1% CLA had no effect on splenocyte PGE_2 production in young or old mice (55). Turek *et al.* (43) suggest that the effect of CLA on PGE_2 production is dependent on the FA composition of the background diet, with the effect of CLA increasing as the ratio of n-6:n-3 PUFA increases. The only study using pure *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers detected no differences in splenocyte PGE_2 production between isomers or compared with controls in mice fed 0.5 wt% CLA (40).

Thus far, data on the effects of CLA on bone metabolism are available from few studies, all of which have used a mixture of isomers. Reduced mineral apposition rate, indicating reduced bone formation following CLA feeding, was reported by Li *et al.* (5). Watkins *et al.* (4), on the other hand, reported increased bone formation (increased IGF-1) in growing chicks fed butterfat (a rich natural source of *cis*-9,*trans*-11 CLA), whereas no effects on bone formation were seen in another study with rats fed 1 wt% CLA (56). We also found no effect for CLA on IGF-1 or another marker of bone formation, plasma osteocalcin. All published studies thus far show no effect for CLA on plasma osteocalcin. Osteocalcin was not affected in rats and chicks fed 1 wt% CLA in the diet (4,5,56) or in a recent human study in healthy males fed 3 g/d *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA (1:1) (57). It has been suggested that the effects of CLA on bone formation may be dependent on the amount of n-6 and n-3 FA in the diet (4).

A bone resorption marker, urinary DPD excretion, was unaffected by CLA in rats fed 1 wt% CLA (53) and in healthy males supplemented with 3 g/d CLA (57). The C-terminal telopeptide α-1 chain of type 1 collagen was used as a marker of resorption in two other studies and was also not affected by CLA (5,57). Our results using a novel resorption marker, TRACP 5b, on the other hand, suggest increased bone resorption at the higher CLA dose (40 mg/kg/d). TRACP 5b activity was correlated with plasma PGE_2 concentrations. PGE_2 is a potent stimulator of bone resorption and the primary prostaglandin affecting bone metabolism (58). These findings should be confirmed in other studies. The effects of diet or CLA on plasma TRACP have not been reported earlier.

In conclusion, no adverse effects were seen with 20 mg/kg/d CLA in growing rats. The novel bone resorption marker used (TRACP 5b) showed increased bone resorption rate on the higher CLA intake level (40 mg/kg/d). Our results show anti-inflammatory effects and enhanced T cell function at *cis*-9,*trans*-11 CLA doses comparable to intakes in exclusively breast-fed infants. If applicable to humans, CLA-induced anti-inflammatory effects could decrease the risk to allergies and atopic diseases, which are increasingly prevalent in infants and young children. *cis*-9,*trans*-11 CLA intake in exclusively breast-fed infants may thus be sufficient to provide beneficial immunological effects. Because it has been proposed that CLA intake during certain periods of growth and development may have long-term benefits and reduce the risk of certain diseases, further studies should be conducted to determine whether vegetable oil–based infant formulas should be supplemented with *cis*-9,*trans*-11 CLA.

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1-*O*-Alkyl-2-(ω-oxo)acyl-*sn*-glycerols from Shark Oil and Human Milk Fat Are Potential Precursors of PAF Mimics and GHB

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ABSTRACT: This study examines the feasibility that peroxidation and lipolysis of 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols (DAGE) found in shark liver oil and human milk fat constitutes a potential source of dietary precursors of platelet activating factor (PAF) mimics and of gamma-hydroxybutyrate (GHB). Purified DAGE were converted into 1-*O*-alkyl-2-acyl-*sn*-glycerols by pancreatic lipase, without isomerization, and transformed into 1-*O*-alkyl-2-oxoacyl-*sn*-glycerols by mild autooxidation. The various core aldehydes without derivatization, as well as the corresponding dinitrophenylhydrazones, were characterized by chromatographic retention time and diagnostic ions by online electrospray mass spectrometry. Core aldehydes of oxidized shark liver oil yielded 23 molecular species of 1-*O*-alkyl-*sn*-glycerols with short-chain *sn*-2 oxoacyl groups, ranging from 4 to 13 carbons, some unsaturated. Autooxidation of human milk fat yielded 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol, as the major core aldehyde. Because diradylglycerols with short fatty chains are absorbed in the intestine and react with cytidine diphosphate-choline in the enterocytes, it is concluded that formation of such PAF mimics as 1-*O*-alkyl-2-(ω-oxo)acyl-*sn*-glycerophosphocholine from unsaturated dietary DAGE is a realistic possibility. Likewise, a C_4 core alcohol produced by aldol-keto reduction of a C_4 core aldehyde constitutes a dietary precursor of the neuromodulator and recreational drug GHB, which has not been previously pointed out.

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Platelet-activating factor (PAF), 1-*O*-alkyl-2-acetyl-*sn*-glycerophosphocholine (GroPCho), is a biologically active phospholipid with diverse physiological and pathological effects in a variety of cells and tissues (1). Gamma-hydroxybutyric acid (GHB) is a simple four-carbon FA with an extraordinary range of physiological and pharmacological effects (2). PAF is known to be enzymatically synthesized by either the remodeling or the *de novo* pathways. PAF mimics, however, are generated by secondary peroxidation of unsaturated 1,2-diacyl-*sn*-GroPCho in cell membranes, which retain a short-chain residue esterified at the *sn*-2 position (3). This short-chain residue may contain either a ω-methyl, ω-aldehyde, ω-alcohol, or ω-carboxyl group for PAF-like activity. Investigations of the biological activities by multiple assays have shown that PAF-like lipids containing an *sn*-1 alkyl ether linkage are more effective than the corresponding *sn*-1 acyl derivatives, and that, in general, the shorter the *sn*-2 chain residue the more active the PAF mimic (4,5). Although it has been suggested (2) that GHB may also arise via lipid peroxidation, the exact mechanism has not been established.

In the present report, we demonstrate the feasibility of metabolic transformation of 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols (DAGE) from shark liver oil and human milk into the corresponding core aldehydes, 1-*O*-alkyl-2-(ω-oxo)acyl-*sn*-glycerols, by mild autooxidation and lipolysis. We have previously shown that short-chain 1,2-diradyl-*sn*-glycerols are absorbed intact in the intestine (6,7) and that exogenous 1,2-diradyl-*sn*-glycerols are incorporated intact into the phosphatidylcholines (PtdCho) (8). We have shown elsewhere (9) that such PAF mimics prepared synthetically induce platelet aggregation and inhibit endothelium-dependent arterial relaxation. We postulate that the C_4 core aldehydes, either as glycerolipids or glycerophospholipids, are reduced to the corresponding C_4 core alcohols by endogenous aldol-keto reductases (10) before release into circulation as GHB. There has been no previous work on the core aldehydes arising from oxidation of alkyldiacylglycerols, although the nonvolatile oxidation products of triacylglycerols have been previously discussed (11–16).

EXPERIMENTAL PROCEDURES

Materials. Crude deep-sea shark liver oil was a gift from Baldur Hjaltason, LYSI Ltd., Reykjavik, Iceland. The lyophilized

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Abbreviations: CapEx, capillary exit; CDP, cytidine diphosphate; DAGE, 1-*O*-alkyl-2,3-diacyl-*sn*-glycerol; DNPH, 2,4-dinitrophenylhydrazine; GE, 1-*O*-alkyl-*sn*-glycerol; GHB, gamma-hydroxybutyric acid; GroPCho, glycerophosphocholine; LC/ESI-MS, LC/electrospray ionization/MS; 2-MAGE, 1-*O*-alkyl-2-acyl-*sn*-glycerol; 3-MAGE, 1-*O*-alkyl-3-acyl-*sn*-glycerol; PAF, platelet-activating factor; PtdCho, phosphatidylcholines (PtdCho); R_f, relative retention factor; RT, retention time.

human milk sample was a gift from Dr. J. Cerbulis of the Eastern Regional Research Center, USDA, Philadelphia, PA. It was one of six milk samples obtained from nursing mothers in the Philadelphia area and used in a collaborative investigation of chloropropanediol diesters in human milk samples (17). The alkyldiacylglycerol composition of these samples ranged from 0.5 to 5 mol%. Linoleic acid, 4-dimethylaminopyridine, and *N*,*N*′-dicyclohexylcarbodiimide were obtained from Sigma-Aldrich (St. Louis, MO). 1-*O*-Octadecyl-*sn*-glycerol was obtained from Fluka (Ronkonkoma, NY). All solvents used were of analytical or HPLC grade.

Preparative TLC. Several preparative TLC systems were employed to purify the various transformation products. All TLC plates were prepared in the laboratory (200 × 200 × 0.25 mm) and activated for 2 h at 110°C before use. System A consisted of silica gel H, developed in hexane/diethyl ether (90:10, vol/vol). System B consisted of silica gel G containing 5% boric acid, developed in hexane/isopropyl ether/acetic acid (50:50:4, by vol). System C consisted of silica gel H, developed in hexane/diethyl ether/acetic acid (80:20:2, by vol). Lipids were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol (18), whereas the core aldehydes were visualized as purple areas after spraying with the Schiff base reagent (19). Migration of a component is given as the relative retention factor (R_f). Lipids and core aldehydes were recovered from the TLC plates by scraping off the gel, extracting it with chloroform/methanol (2:1, vol/vol), washing with water, drying over anhydrous sodium sulfate, evaporating under nitrogen, and dissolving in chloroform/methanol (2:1, vol/vol). The 2,7-dichlorofluorescein was removed with 1% ammonium hydroxide.

GLC. Injections were made at 100°C, and after 30 s the oven temperature was programmed at 20°C/min to either 130°C (FAME) or 180°C (diacetyl-derivatized 1-*O*-alkyl-*sn*-glycerols (GE)), and then to 240°C at 5°C/min (18). The GLC system consisted of a polar capillary column (SP 2380, 15 m × 0.32 mm i.d., Supelco, Mississauga, ON) installed in a Hewlett-Packard (Palo Alto, CA) Model 5880 gas chromatograph equipped with a flame ionization detector. Hydrogen was used as carrier gas at 3 psi. FAME and diacetyl-GE were identified on the basis of retention times (RT) compared with commercially available external reference compounds.

HPLC. Reversed-phase HPLC was performed with a Hewlett-Packard Model 1090 liquid chromatograph (Palo Alto, CA) using an HP ODS Hypersil C_{18} column (5 μm; 200 × 2.1 mm i.d.; Hewlett-Packard, Palo Alto, CA) and eluted isocratically with 100% Solvent A (methanol/water/30% ammonium hydroxide, 88:12:0.5, by vol) for 3 min, followed by a linear gradient to 100% Solvent B (methanol/hexane/30% ammonium hydroxide, 88:12:0.5, by vol) in 25 min, which was kept for another 6 min (20). Kim *et al.* (20) washed the HPLC column with 0.1 M ammonium acetate at 0.5 mL/min for 5 min at the end of each run and did not observe any ill effects on the performance of column or the quality of the mass spectra. When 2,4-dinitrophenylhydrazine (DNPH) derivatives were analyzed, the effluent was led through a UV detector (358 nm) installed before the mass spectrometer. The flow was 0.4 mL/min.

Electrospray ionization MS (ESI-MS). Reversed-phase HPLC with online electrospray ionization MS (LC/ESI-MS) was performed by admitting the entire HPLC column effluent into a Hewlett-Packard Model 5988B quadrupole mass spectrometer (Palo Alto, CA) equipped with a nebulizer-assisted electrospray interface (Hewlett-Packard Model 59987A, Palo Alto, CA) as previously described (19). Nitrogen was used as both nebulizing (60 psi) and drying gas (60 psi, 270°C). Capillary voltage was set at 4 kV, the endplate voltage was 3.5 kV, and the cylinder voltage was 5 kV in the positive mode of ionization. In the negative mode, the values were −3.5 kV, −3 kV, and −3.5 kV, respectively. Both negative and positive ESI spectra were taken in the mass range 300–1100 amu. The capillary exit (CapEx) was set at 120 and −120 V in the positive and negative ion mode, respectively.

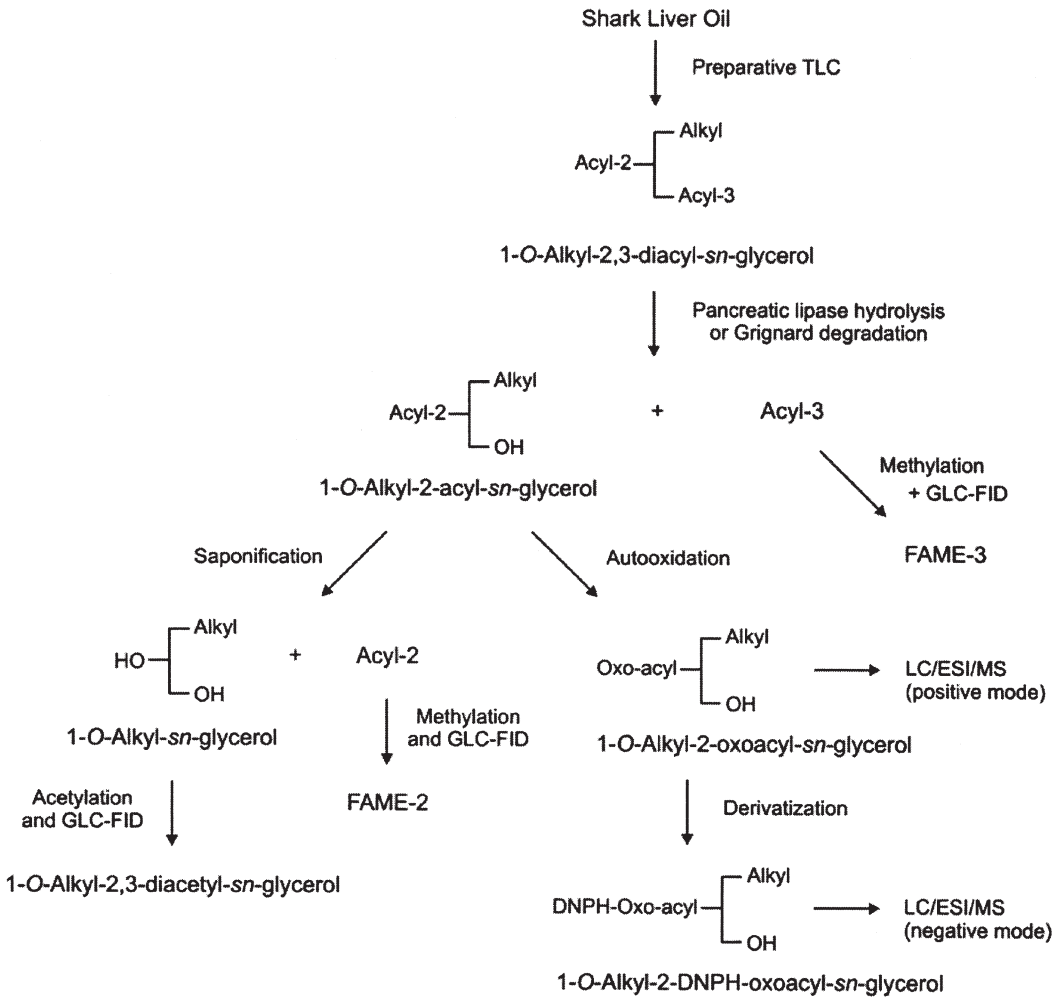
Preparation of DAGE from shark liver oil and human milk. A total lipid extract of freeze-dried human milk was prepared as previously described (21). DAGE was recovered from the human milk lipid extract and the shark liver oil by preparative double one-dimensional TLC (system A). The purified DAGE was subjected to regiospecific analysis to reveal the *sn*-1-*O*-alkyl-, *sn*-2 acyl-, and *sn*-3 acyl-chain composition and distribution. The complete procedure is outlined in Scheme 1.

Hydrolysis with pancreatic lipase and Grignard degradation. Purified DAGE of shark liver oil and human milk were hydrolyzed by digestion with diethyl ether pre-extracted pancreatic lipase (22). The digestion was performed in the presence of gum arabic for 30 min, and the digestion products were extracted with diethyl ether. Alternatively, the purified DAGE were deacylated by Grignard degradation (23) in order to verify the results obtained from pancreatic lipase digestion. The degradation products were resolved and recovered by TLC (system B).

Preparation of FAME and diacetyl-GE. Purified fractions of DAGE, 2-MAGE, and 3-MAGE originating from shark liver oil were treated with 6% H_2SO_4 in methanol for 2 h at 80°C to produce FAME and GE. After the reaction, the lipids were extracted twice with chloroform. GE and FAME were resolved and recovered by preparative TLC (system C). Purified GE was derivatized to diacetyl-GE for 30 min at 80°C with acetic anhydride/pyridine (1:1, vol/vol; 75 μL). The profiles of FAME and diacetyl-GE were determined by GLC.

Autooxidation of 2-MAGE. Mild peroxidation was performed by flushing the purified 2-MAGE from either shark liver oil or human milk in a tube with oxygen, capping, and heating at 80°C for 3 h. The peroxidized 2-MAGE was analyzed by reversed-phase LC/ESI-MS.

Preparation of DNPH derivatives. Aldehyde preparations of oxidized 2-MAGE were derivatized by reaction with DNPH in the dark (0.5 mg in 1 mL 1 N HCl) for 2 h at room temperature and 1 h at 4°C (24). The DNPH derivatives were extracted with chloroform/methanol (2:1, vol/vol), dried over anhydrous sodium sulfate, evaporated under a stream of nitrogen, dissolved in chloroform/methanol (2:1, vol/vol), and analyzed by



SCHEME 1. Flow sheet for the regiospecific analysis of DAGE from shark liver oil and the procedure for isolation and characterization of core aldehydes from oxidized and digested DAGE.

reversed-phase LC/ESI-MS. Derivatization with DNPH, therefore, increased the detection limit for the core aldehydes by MS, and further provided additional ions for characterization of the alkyl ether core aldehydes, as well as an opportunity to monitor the components by UV detection at 358 nm.

Preparation of reference 1-O-octadecyl-2-(9-oxo)-nonanoyl-sn-glycerol. The esterfication of 1-*O*-octadecyl-*sn*-glycerol with linoleic acid was performed by the carbodiimide-mediated process (25). Linoleic acid (75 μmol), 1-*O*-octadecyl-*sn*-glycerol (100 μmol), and 4-dimethyl-aminopyridine (10 μmol) were dissolved in dry n-hexane. This solution was added to a suspension of *N*,*N*′-dicyclohexylcarbodiimide (100 μmol) in dry n-hexane and shaken vigorously for 17 h at room temperature. After filtration, solvent was evaporated under nitrogen, and the residue was purified by preparative TLC (system B). We have previously reported the LC/ESI-MS analysis of this and other related synthetic neutral ether lipids (26).

The synthesized and purified 1-*O*-octadecyl-2-octadecadienoyl-*sn*-glycerol was subjected to triphenylphosphine reductive ozonization as previously described (19). The resulting reference core aldehyde, 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol (R_f = 0.11), was purified by preparative TLC (system B) and analyzed by reversed-phase LC/ESI-MS, and its identity was established on basis of RT, averaged mass spectrum, and fragmentation pattern.

RESULTS

Isolation of DAGE. Preparative TLC resolved the crude shark liver oil into six bands, which corresponded to monoacyl (monoradyl) glycerols (R_f = 0.01), free cholesterol (R_f = 0.13), triacylglycerol (R_f =0.37), DAGE (R_f = 0.55), cholesteryl esters (R_f = 0.93), and squalene (R_f = 0.97). The TLC bands were scraped off the plate and analyzed by high-temperature GLC, which indicated that the DAGE made up 55% of the shark liver oil. Reversed-phase LC/ESI-MS indicated that DAGE was composed of at least 50 species eluting between 10 and 37 min (chromatogram not shown). Similarly, the DAGE content of human milk was estimated to be approximately 1% of total fat and was made up of numerous species, of which only a few were abundant.

Regiospecific analysis of DAGE (26). The purified DAGE were subjected to a regiospecific analysis to reveal the *sn*-1-*O*-

alkyl, *sn*-2-acyl, and *sn*-3-acyl chain composition and distribution, which were determined by TLC (system B) and GLC, following pancreatic lipase hydrolysis and Grignard degradation.

Pancreatic lipase hydrolysis of the DAGE yielded 1-*O*-alkyl-2-acyl-*sn*-glycerols (2-MAGE, R_f = 0.32) as the major product (97%) and 1-*O*-alkyl-3-acyl-*sn*-glycerol (3-MAGE, R_f = 0.41) as the minor product (3%), along with GE (R_f = 0.05), free FA (R_f = 0.66), and original DAGE (R_f = 0.90).

The high recovery of 2-MAGE compared with 3-MAGE is consistent with the resistance of the *sn*-1 ether linkage to the action of most enzymes, and furthermore indicates a very low rate of isomerization and a low affinity of the pancreatic lipase for the *sn*-2-position of DAGE. The non-selective Grignard degradation yielded the 2-MAGE and 3-MAGE in equal amounts together with GE and the free FA as the tertiary alcohols (Grignard reaction products). The DAGE, 2-MAGE, and 3-MAGE fractions recovered from the pancreatic lipase digestion and Grignard degradation were treated with sulfuric acid/methanol to produce FAME and glyceryl ethers (GE), which were resolved by preparative TLC (system C). The purified GE were converted into the diacetyl GE by reaction with acetic anhydride and pyridine, and the FAME and the GE acetates were identified and quantified by GLC. Reversed-phase LC/ESI-MS analysis of 2-MAGE isolated from the shark liver oil following pancreatic lipolysis of DAGE showed a total of 49 species, of which 20 species were abundant, eluting between 10 and 26 min (chromatogram not shown).

Table 1 gives the regiospecific distribution of the fatty chains of shark liver oil DAGE as determined by GLC analysis of products of pancreatic lipolysis and Grignard degradation. The predominant *sn*-1-*O*-alkyl fatty chains were the monounsaturated alcohols (18:1n-9, 54.9%, and 16:1n-7, 12.2%) and saturated alcohols (16:0, 11.2%), with much smaller amounts of a diunsaturated alcohol (18:2n-6, 1%). Small amounts of odd-carbon saturated and monounsaturated fatty alcohols were also detected. Pancreatic lipase digestion and Grignard degradation gave similar FA profiles and selectivity for the *sn*-2- and *sn*-3-positions. The most abundant *sn*-2-FA were 16:0, 16:1n-7, 18:1n-9, 20:1n-9, 22:1n-11/13, 22:5n-3, and 22:6n-3, with 18:1n-9 accounting for more than 50% of the total. The most abundant *sn*-3-FA were 16:0, 16:1n-7, 18:0, 18:1n-9, 20:1n-9, 22:1n-11/13, and 22:4n-3. The FA 18:1n-9, 22:5n-3, and 22:6n-3 were preferentially associated with the *sn*-2-position, whereas 18:0, 20:1n-9, 22:1n-11/13, and 22:4n-3 were mostly in the *sn*-3-position.

LC/ESI-MS characterization of reference core aldehydes. The identities of all synthetic neutral ether lipids were established by combined TLC and LC/ESI-MS analysis. The 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol was produced in high yield and purity by reductive ozonization of 1-*O*-octadecyl-2-(9-*cis*,12-*cis*)-octadecadienoyl-*sn*-glycerol. Figure 1A shows the total LC/ESI-MS positive ion current profile (CapEx +120 V) of synthetic 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol (RT = 14.8 min). Figure 1B shows the full mass spectrum averaged over the entire peak with eight major ions being observed. The assignments for the observed ions, their corre-

TABLE 1
Regiospecific Distribution (mol%) of FA of Shark Liver Oil DAGE as Determined by GLC-FID After Pancreatic Lipase Hydrolysis or Grignard Degradation[a]

		sn-2 Acyl		*sn*-3 Acyl	
Fatty chain	*sn*-1 Alkyl	Lipase	Grignard	Lipase	Grignard
12:0	1.0	nd	nd	nd	nd
14:0	2.3	1.0	1.4	0.7	0.8
15:0	0.4	0.3	0.3	0.1	0.2
16:0	11.2	18.4	18.8	15.9	19.5
16:1n-7	12.2	4.7	4.9	3.2	3.2
16:2n-4	nd	0.7	0.7	0.9	1.0
17:0	0.6	nd	nd	nd	nd
17:1	2.2	nd	nd	nd	nd
18:0	2.9	1.2	1.7	4.6	3.7
18:1n-9	54.9	51.8	48.9	21.3	16.9
18:1n-7	4.6	nd	0.3	5.8	6.6
18:2n-6	1.0	0.8	0.8	0.7	0.2
18:3n-3	nd	0.3	0.4	0.4	0.3
18:4n-3	nd	0.2	0.2	0.2	0.1
19:0	0.6	nd	nd	nd	nd
20:1n-9	2.6	6.1	5.5	11.1	12.4
20:2n-6	nd	0.1	0.2	0.3	0.3
20:4n-6	nd	nd	0.1	nd	nd
20:5n-3	nd	0.1	0.3	0.5	0.2
22:1n-11/13	0.2	3.7	3.4	18.0	21.0
22:4n-3	nd	0.2	0.2	5.9	7.4
22:5n-3	nd	1.6	1.4	0.5	0.4
22:6n-3	nd	4.1	3.3	1.5	0.8
24:1n-3	nd	0.7	0.9	0.3	0.2

[a]Average of two determinations. nd, not detected.

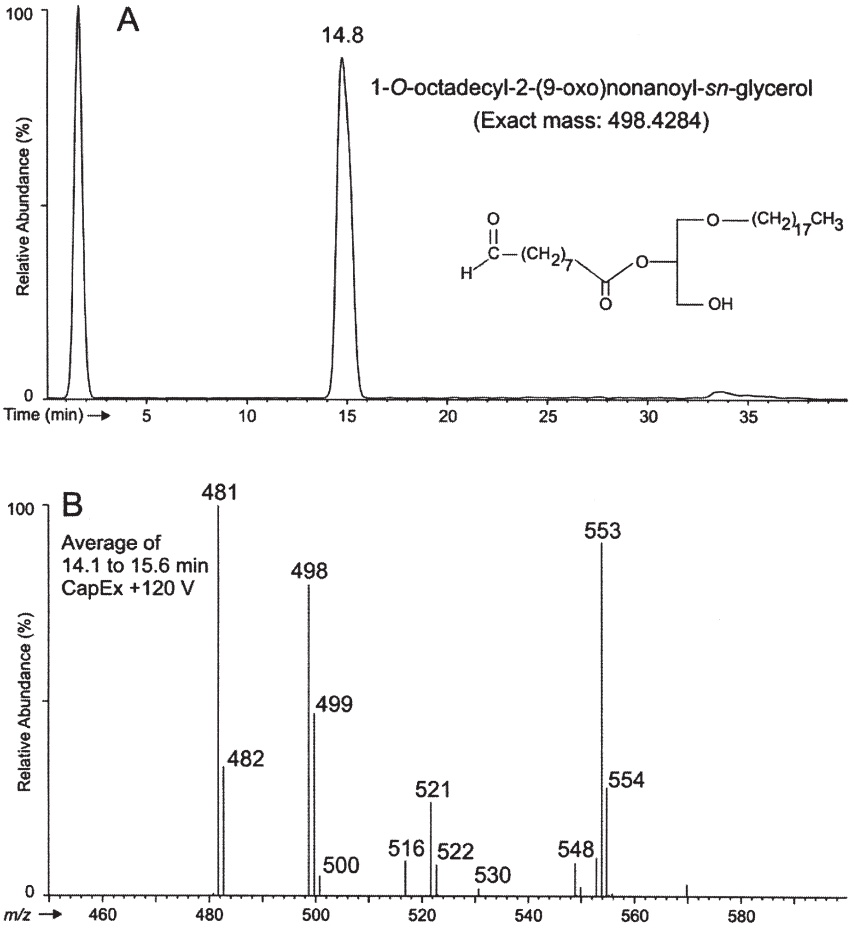


FIG. 1. Reversed-phase LC/ESI-MS analysis of synthetic 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol. (A) Total positive ion current profile. (B) Full mass spectrum averaged over the entire peak, zoomed to *m/z* 450–600, in (A). All of the ions detected in the spectrum were assigned to the original reference compound. Note the high abundance for the *m/z* 499 ion relative to *m/z* 498, suggesting that the *m/z* 499 ion is not entirely composed of the ^{13}C isotopic ion of *m/z* 498. The LC/ESI-MS analysis showed that the optimal diagnostic ion of the 1-*O*-alkyl-2-(ω-oxo)acyl-*sn*-glycerols is the $[M - 17]^+$ ion, which in (B) corresponds to *m/z* 481. Additional details are given in Table 2, Scheme 2, and the text.

sponding ^{13}C isotopic components, and their relative abundances are presented in Table 2. The proposed molecular structures for these assigned ions are shown in Scheme 2. Sodium and ammonium adducts are common features of ESI, and it is also known that aldehydes in methanol solutions, as encountered in the mobile phase, are converted to the corresponding neutral hemi-acetal form (a methanol adduct).

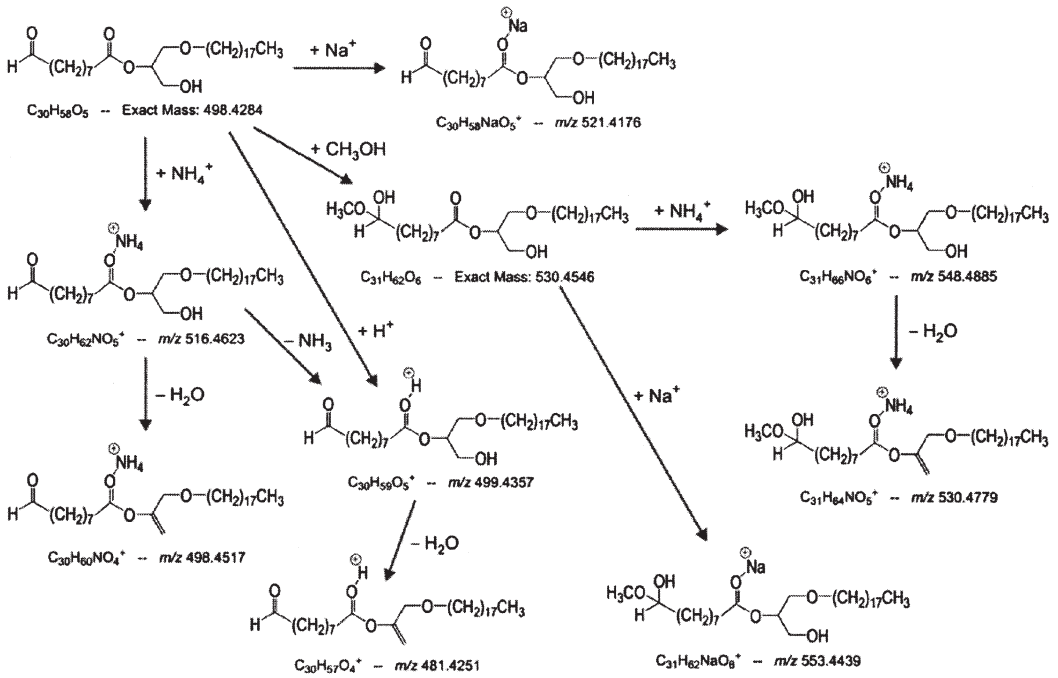
The presence of an ion at *m/z* 498.70 corresponding to the $[M]^+$ ion was unexpected and only observed in the samples with the underivatized monoalkylglycerols containing a free aldehyde ester group. We confirmed this observation with the corresponding 3-isomer reference compound, 1-*O*-octadecyl-3-(9-oxo)nonanoyl-*sn*-glycerol (data not shown). It would be expected that the observed ion would have *m/z* 499.75 corresponding to the protonated molecular ion $[M + H]^+$. As indicated in Table 2 and Scheme 2, we propose that the $[M]^+$ ion at *m/z* 498.70 arises from a dehydrated ammonium adduct, $[M + NH_4 - H_2O]^+$, and not directly from the ionization as a radical cation, $[M]^{+\cdot}$. Another unexpected observation was the relatively high abundance (59%) of the *m/z* 499.75 ion compared with the *m/z* 498.70 ion, which is much higher than the calculated contribution of the ^{13}C isotope (32%) for this compound. The other ions have the ^{13}C contribution showing the expected ~32% relative abundance (Table 2). This suggests that the peak at *m/z* 499.75 is actually composed of two different species, $[(M + {}^{13}C_1) + NH_4 - H_2O]^+$ and the protonated molecular ion, $[M + H]^+$. As indicated in Table 2 and Scheme 2, we propose that the $[M + H]^+$ ion can also arise indirectly from the neutral loss of ammonia from the molecular ammonium adduct, $[M + NH_4 - NH_3]^+$.

Due to the lengthy and complicated ion assignment and the multiple possible pathways of ion formation, we have decided to describe the ions as mass difference from the exact mass of the 1-*O*-alkyl-2-(ω-oxo)-*sn*-glycerol, that is, $[M \pm X]^+$.

TABLE 2
Mass Spectrometric Analysis of Synthetic 1-*O*-Octadecyl-2-(9-Oxo)Nonanoyl-*sn*-Glycerol: Assignment of Detected Ions Including Corresponding ^{13}C Isotopic Ions and Their Relative Abundance[a]

Ion ID	Ion (m/z) (Rel. Ab.)[b]	^{13}C Ion (m/z)	(Rel. Ab.)	Ion assignment
$[M - 17]^+$	481.65 (100%)	+1	482.65 (33%)	$[M + H - H_2O]^+$ and/or $[M + NH_4 - NH_3 - H_2O]^+$
$[M]^+$	498.70 (80%)	+1	499.75 (32%)[c]	$[M + NH_4 - H2O]+$
$[M + 1]^+$	499.75 (21%)[d]	+1	500.75 (24%)[e]	$[M + H]^+$ and/or $[M + NH_4 - NH_3]^+$
$[M + 18]^+$	516.70 (9%)			$[M + NH_4]^+$
$[M + 23]^+$	521.70 (24%)	+1	522.70 (33%)	$[M + Na]^+$
$[M + 32]^+$	530.70 (2%)			$[M + CH_3OH + NH_4 - H_2O]^+$
$[M + 50]+$	548.70 (9%)	+1	549.80 (31%)	$[M + CH_3OH + NH_4]+$
$[M + 55]^+$	553.85 (91%)	+1	554.75 (31%)	$[M + CH_3OH + Na]+$

[a]Average of four mass spectra.
[b]Rel. Ab., relative ion abundance.
[c]Calculated Rel. Ab. based on expected ^{13}C contribution and the average of the other detected ^{13}C isotopic ions.
[d]Calculated Rel. Ab. as the total ion abundance for *m/z* 499.75 subtracted the contribution from the ^{13}C isotopic ion of *m/z* 498.70; calculated Rel. Ab. based on the calculated ion abundance. Additional details are given in Figure 1, Scheme 2, and the text.
[e]Calculated Rel. Ab. based on the calculated ion abundance for m/z 499.75 [M+1]+, see [d].



SCHEME 2. Proposed ionization and formation of various ions in the spectrum of synthetic 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol. Additional details are given in Figure 1, Table 2, and the text.

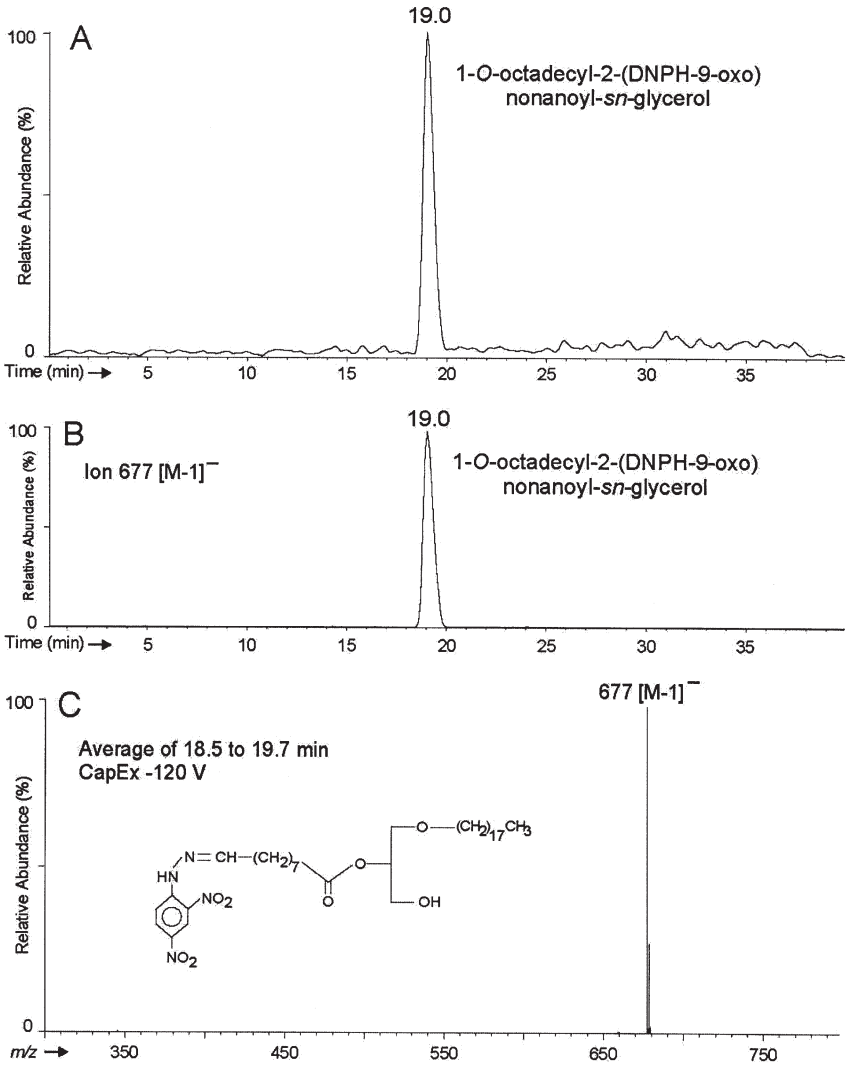


FIG. 2. Reversed-phase LC/ESI-MS analysis of synthetic and DNPH derivatized 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol. (A) Total negative ion current profile. (B) Reconstructed single-ion chromatogram of the *m/z* 677 $[M - 1]^-$ ion. (C) Full mass spectrum averaged over the entire peak, zoomed to *m/z* 300–800, in (A). The diagnostic ion of 1-*O*-alkyl-2-(DNPH-oxo)acyl-*sn*-glycerols is thus the $[M - 1]^-$ ion.

The best diagnostic ion of 1-*O*-alkyl-2-oxoacyl-*sn*-glycerols under our experimental LC/ESI-MS conditions was the $[M - 17]^+$ ion at *m/z* 481.65, which we propose arises from the dehydration of the *m/z* 498.70 $[M + 1]^+$ ion (Fig. 1B, Table 2, and Scheme 2).

Further identification of 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol was performed by derivatization with DNPH. Figure 2 shows the total LC/ESI-MS negative ion current profile with CapEx at −120 V of the DNPH-derivatized 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol (RT 19.0 min, Fig. 2A) along with the reconstructed single-ion chromatogram (Fig. 2B) for the $[M - 1]^-$ ion, and the full mass spectrum (Fig. 2C) averaged over the entire peak. As demonstrated in Figure 2C, 1-*O*-octadecyl-2-(DNPH-9-oxo)nonanoyl-*sn*-glycerol was characterized by the deprotonated molecular ion, $[M - 1]^-$ at *m/z* 677, which was used as the diagnostic ion.

Characterization of 2-MAGE core aldehydes in autooxidized shark liver oil. Figure 3 shows the total positive ion current profile of the 1-*O*-alkyl-2-acyl-*sn*-glycerols before autooxidation (Fig. 3A) and after 3 h autooxidation (Fig. 3B) of shark liver oil 2-MAGE. The resulting ether core aldehydes (i.e., 1-*O*-alkyl-2-(ω-oxo)acyl-*sn*-glycerols) eluted with retention times ranging from 9 to 15 min, the hydroperoxides (i.e., 1-*O*-alkyl-2-(hydroperoxy)acyl-*sn*-glycerols) and other oxidation products with retention times ranging from 5 to 25 min, and the non-oxidized 1-*O*-alkyl-2-acyl-*sn*-glycerols with retention times from 25 to 35 min. The core aldehydes made up about 5%, and the other oxidation products about 40%, of the total peak area (Fig. 3B). Figure 3 also shows the reconstructed single-ion chromatograms for the $[M - 17]^+$ ion of 1-*O*-octadecenyl-2-(4-oxo)butyroyl-*sn*-glycerol (Fig. 3C) and 1-*O*-octadecenyl-2-(9-oxo)nonanoyl-*sn*-glycerol (Fig. 3D). Several 1-*O*-

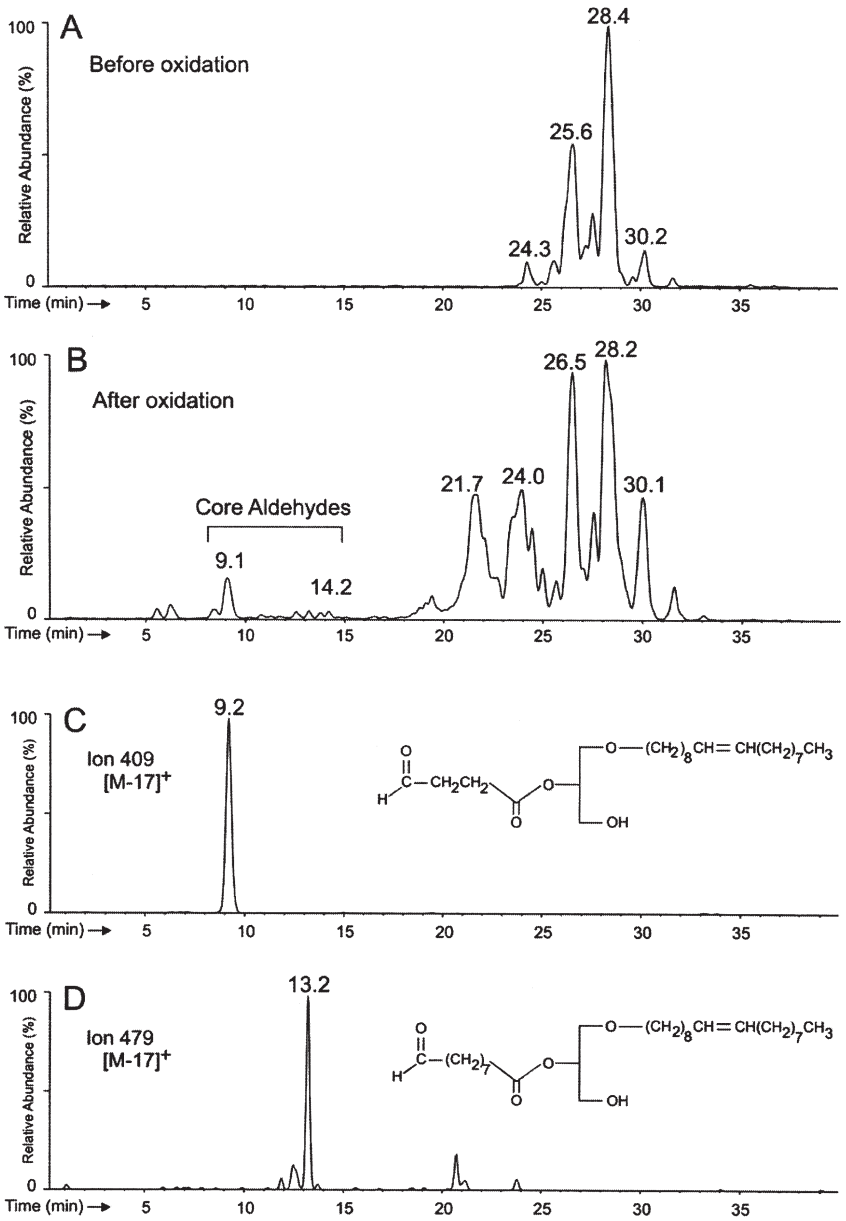


FIG. 3. Reversed-phase LC/ESI-MS analysis of 1-*O*-alkyl-2-acyl-*sn*-glycerols isolated from shark liver oil before and following oxidation. (A) and (B) Total positive ion current profiles of non-oxidized and autooxidized (80°C for 3 h in oxygen atmosphere) 1-*O*-alkyl-2-acyl-*sn*-glycerols, respectively. (C) and (D) reconstructed single-ion chromatograms of the *m/z* 409 and 479 $[M - 17]^+$ diagnostic ion of 1-*O*-octadecenyl-2-(4-oxo)butyroyl-*sn*-glycerol and 1-*O*-octadecenyl-2-(9-oxo)nonanoyl-*sn*-glycerol, respectively.

alkyl-2-oxoacyl-*sn*-glycerols were identified on the basis of the $[M - 17]^+$ and $[M]^+$ ions (Table 3). The major ether core aldehydes corresponded to 18:1-4:0Ald (20%), 16:1-9:0Ald (18:1-7:0Ald) (10%), 16:0-9:0Ald (2%), 16:0-10:1 (17:1-9:0Ald) (2%), 18:1-9:0Ald (20%), and 18:1-10:1Ald (7%).

Figure 4 shows the total LC/ESI-MS negative ion current profile (Fig. 4A) of DNPH-derivatized and purified 1-*O*-alkyl-2-oxoacyl-*sn*-glycerol, derived from shark liver oil DAGE, along with selected, reconstructed single-ion mass chromatograms for the $[M - 1]^-$ ion of 1-*O*-hexadecenyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerol (Fig. 4B), 1-*O*-hexadecyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerol (Fig. 4C), 1-*O*-octadecenyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerol (Fig. 4D), 1-*O*-octadecenyl-2-(DNPH-7-oxo)heptanoyl-*sn*-glycerol (Fig. 4E), 1-*O*-octadecenyl-2-(DNPH-9-oxo)nonanoyl-*sn*-glycerol (Fig. 4F), and 1-*O*-octadecyl-2-(DNPH-9-oxo)nonanoyl-*sn*-glycerol (Fig. 4G), which all were eluted over the time period of 10 to 20 min.

Table 4 lists the identified molecular species along with the uncorrected peak areas attributed to them. The $[M - 1]^-$ ion provides only the molecular weight of the compound. Further

TABLE 3
Identification of Molecular Species of 1-*O*-Alkyl-2-Oxoacyl-*sn*-Glycerols, Produced by Autooxidation of Pancreatic Lipase Treated Shark Liver Oil DAGE and Estimated by LC/ESI-MS[a]

Molecular species		1-*O*-Alkyl-2-oxoacyl-*sn*-glycerol		
Carbon number	Tentative identity	RT (min)	$[M]^+$ (m/z)	$[M - 17]^+$ (m/z)
22:1 Ald	18:1-4:0 Ald	9.2	426	409
25:1 Ald	16:1-9:0 Ald 18:1-7:0 Ald	11.0	nd	451
25:0 Ald	16:0-9:0 Ald	11.3	nd	453
26:1 Ald	16:0-10:1 Ald 17:1-9:0 Ald	11.7	nd	465
27:1 Ald	18:1-9:0 Ald	12.6	496	479
28:2 Ald	18:1-10:1 Ald	13.2	508	491

[a]Two analyses. RT, retention time; Ald, aldehyde; nd, not detected.

confirmation of the structure was obtained from the linear correlation ($R = 0.982$) obtained between retention time and molecular mass among the 1-*O*-alkyl-2-DNPH-oxoacyl-*sn*-glycerols, when the latter was calculated as the total carbon number minus one carbon per double bond (Table 4). This elution factor makes it possible to calculate the relative retention times of unknowns with considerable accuracy, which helped to choose among likely structures represented by the same molecular mass within the reversed-phase LC/ESI-MS profile. Further characterization of the molecular species was obtained from knowledge of the possible formation of esterified aldehyde residues and the ratios of the corresponding *sn*-1 and *sn*-2 fatty chain moieties (Tables 1 and 4). For example, the masses at *m/z* 577 and 579 ($[M - 1]^-$ ions) can only represent the 1-*O*-hexadecenyl- and 1-*O*-hexadecyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerols (Figs. 4B and 4C), respectively. The ion at *m/z* 605 $[M - 1]^-$, however, could represent both the 1-*O*-octadecenyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerol and the 1-*O*-pentadecyl-2-(DNPH-7-oxo)heptanoyl-*sn*-glycerols (Table 4 and Fig. 4D), but on the basis of the much higher abundance of *sn*-1 alkyl 18:1n-9 in comparison with 15:0 (Table 1), it is obvious that the peak area mainly represents the 1-*O*-octadecenyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerol. The alternative identities of the species are written in parentheses in Table 4.

Characterization of 2-MAGE core aldehydes in autooxidized human milk. The small DAGE fraction isolated from human milk fat yielded a 1-*O*-hexadecylglycerol and oleic acid from the *sn*-2- and *sn*-3-positions as the major fatty chains. Figure 5 shows the total LC/ESI-MS negative ion current profile (Fig. 5A) of DNPH-derivatized 1-*O*-alkyl-2-oxoacyl-*sn*-glycerols, derived from human milk DAGE, along with the reconstructed single-ion mass chromatogram (Fig. 5B) for the $[M - 1]^-$ ion of 1-*O*-octadecyl-2-(DNPH-9-oxo)nonanoyl-*sn*-glycerol (RT = 18.8 min), and the full mass spectrum (Fig. 5C) averaged over the entire peak. This was the only ether core aldehyde characterized from human milk.

DISCUSSION

The major source of PAF-like lipids (or mimics/analogues) is the unregulated oxidative modification of cellular and plasma phospholipids. There have been numerous reports on isolation of the PAF-like lipids containing a long-chain fatty ester in the *sn*-1-position and a short-chain (ω-oxo)acyl group in the *sn*-2-position of PtdCho (27). The immediate precursors of these mimics are the 1,2-diacyl- and 1-*O*-alkyl-2-acyl-*sn*-GroPCho with saturated acyl and alkyl chains in the *sn*-1-position. Among these, the most frequently reported are 1-hexadecanoyl- and 1-octadecanoyl-2-(5-oxo)pentanoyl-*sn*-GroPCho, which are generated from the corresponding arachidonates (27). The corresponding *sn*-1 alkyl ether derivative, which is structurally more closely related to PAF, has been studied less frequently due to the more limited supply of potential precursors, although it was the first PAF analogue identified (27) and both DAGE and 1-*O*-alkyl-2-acyl-*sn*-glycerophospholipids are present in significant amounts of tissue lipids (28). The present study demonstrates the feasibility of generating PAF analogue precursors in the intestine by lipolysis and peroxidation of the DAGE, which constitute a significant proportion of dietary fats such as shark liver oil and milk fat. In addition, this study has recognized certain unusual features of 1-*O*-alkyl-2-(ω-oxo)acyl-*sn*-glycerols as precursors of PAF analogues. The 1,2-diradyl-*sn*-glycerol moieties generated from DAGE of shark liver oil were characterized by the presence of sites of unsaturation in both fatty chains. The peroxidation would, therefore, be expected largely to affect the 18:2n-6, 18:3n-3, and especially the 22:5n-3 and 22:6n-3, which would be anticipated to yield C_4 to C_9 core aldehydes in combination with both saturated and monounsaturated alkyl chains in the *sn*-1-position. The present results confirm that peroxidation of the monounsaturated 1-*O*-alkyl chains was limited during the mild conditions as previously described (29).

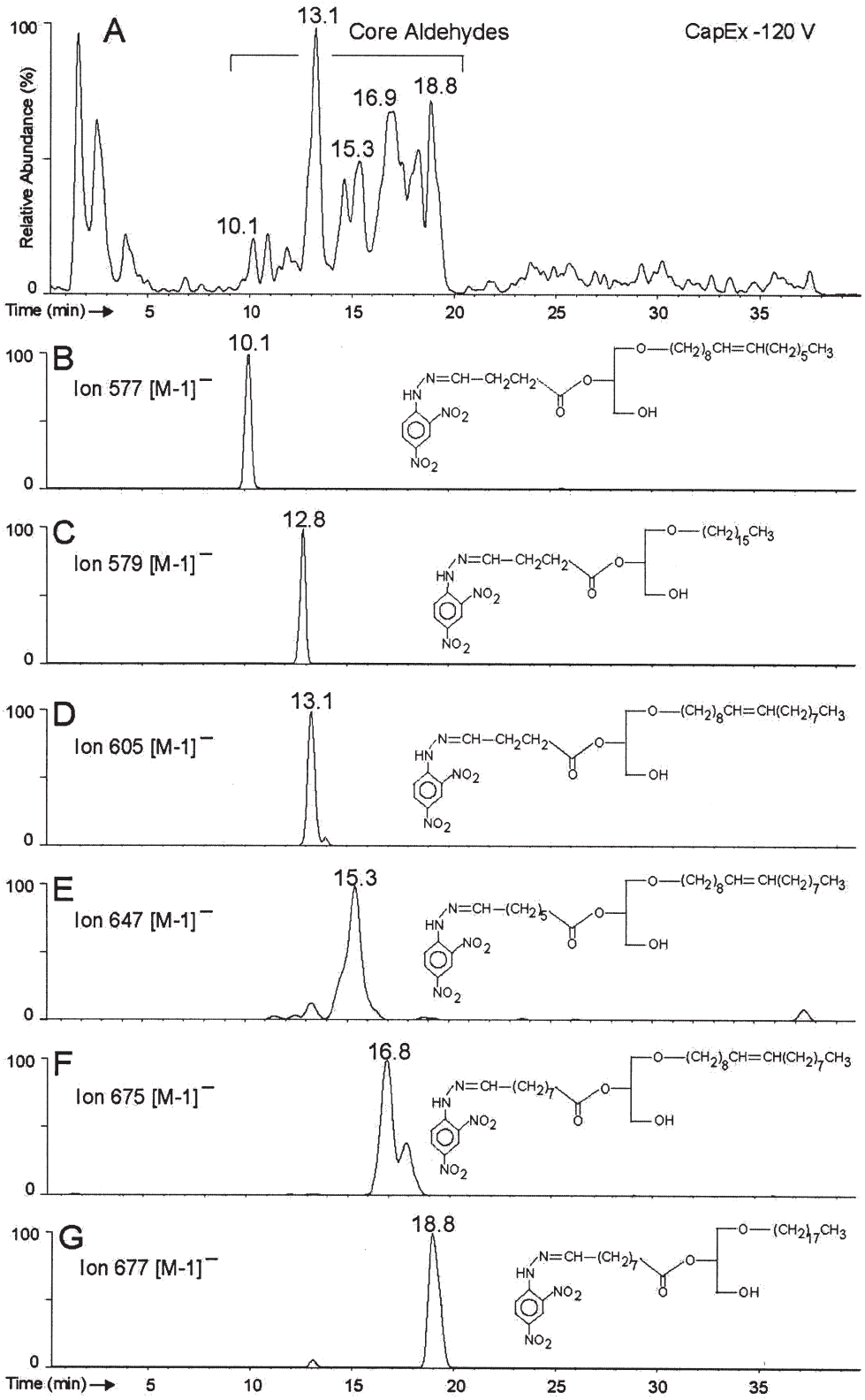


FIG. 4. Reversed-phase LC/ESI-MS analysis of autooxidized and DNPH derivatized 1-*O*-alkyl-2-oxoacyl-*sn*-glycerols from shark liver oil. (A) Total negative ion current profile of 1-*O*-alkyl-2-(DNPH-oxo)acyl-*sn*-glycerols. (B–G) reconstructed single-ion mass chromatograms of *m/z* 577, 579, 605, 647, 675, and 677 $[M - 1]^-$ diagnostic ions of 1-*O*-hexadecenyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerol, 1-*O*-hexadecyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerol, 1-*O*-octadecenyl-2-(DNPH-4-oxo)butyroyl-*sn*-glycerol, 1-*O*-octadecenyl-2-(DNPH-7-oxo)heptanoyl-*sn*-glycerol, 1-*O*-octadecenyl-2-(DNPH-9-oxo)nonanoyl-*sn*-glycerol, and 1-*O*-octadecyl-2-(DNPH-9-oxo)nonanoyl-*sn*-glycerol, respectively.

TABLE 4
Composition of Molecular Species of DNPH-Derivatized 1-*O*-Alkyl-2-Oxoacyl-sn-Glycerols Produced by Autooxidation of Pancreatic Lipase Treated Shark Liver Oil DAGE as Estimated by LC/ESI-MS[a]

Molecular species		1-*O*-Alkyl-2-DNPH-oxoacyl-*sn*-glycerol			
Carbon number	Tentative identification	ECN	RT (min)	$[M-1]^-$ (m/z)	Peak area
20:1 Ald	16:1-4:0 Ald	19	10.1	577	4.0
20:0 Ald	16:0-4:0 Ald	20	12.8	579	7.6
22:2 Ald	18:2-4:0 Ald	20	11.8	603	0.1
21:0 Ald	14:0-7:0 Ald 17:0-4:0 Ald	21	13.6	593	0.3
22:1 Ald	18:1-4:0 Ald (15:0-7:1 Ald)	21	13.1	605	21.9
22:0 Ald	18:0-4:0 Ald (15:0-7:0 Ald)	22	14.2	607	0.6
23:1 Ald	16:1-7:0 Ald 16:0-7:1 Ald	22	13.7	619	0.3
24:2 Ald	14:0-10:2 Ald 16:0-8:2 Ald 17:1-7:1 Ald	22	14.4	631	0.2
23:0 Ald	14:0-9:0 Ald 16:0-7:0 Ald †	23	15.1	621	2.2
24:1 Ald	17:1-7:0 Ald 20:1-4:0 Ald (17:0-7:1 Ald)†	23	14.6	633	4.7
25:2 Ald	18:1-7:1 Ald (14:0-11:2 Ald) (18:2-7:0 Ald)	23	15.0	645	2.2
25:1 Ald	18:1-7:0 Ald 16:1-9:0 Ald (18:0-7:1 Ald)	24	15.3	647	11.3
25:0 Ald	16:0-9:0 Ald (18:0-7:0 Ald)	25	16.6	649	2.4
26:1 Ald	16:0-10:1 Ald 17:1-9:0 Ald	25	16.5	661	1.7
27:2 Ald	17:1-10:1 Ald 20:1-7:1 Ald (14:0-13:2 Ald) (16:0-11:2 Ald) (18:2-9:0 Ald)	25	16.1	673	1.8
26:0 Ald	15:0-11:0 Ald 17:0-9:0 Ald 19:0-7:0 Ald	26	17.5	663	0.1
27:1 Ald	18:1-9:0 Ald (20:1-7:0 Ald)	26	16.9	675	18.7
28:2 Ald	18:1-10:1 Ald (16:1-12:1 Ald) (18:0-10:2 Ald)	26	17.4	687	6.9
27:0 Ald	18:0-9:0 Ald 16:0-11:0 Ald	27	18.8	677	6.9
28:1 Ald	16:0-12:1 Ald 18:0-10:1 Ald	27	17.4	689	0.4
29:2 Ald	16:0-13:2 Ald (19:0-10:2 Ald)	27	18.2	701	4.5
29:1 Ald	18:1-11:0 Ald 20:1-9:0 Ald (22:1-7:0 Ald)	28	18.9	703	1.3
30:2 Ald	18:1-12:1 Ald 20:1-10:1 Ald	28	19.0	715	0.1

[a]Average of two determinations. ECN, equivalent carbon number; RT, retention time; Peak area, percent of cumulative peak area of recovered $[M-1]^-$ ions; Ald, aldehyde.

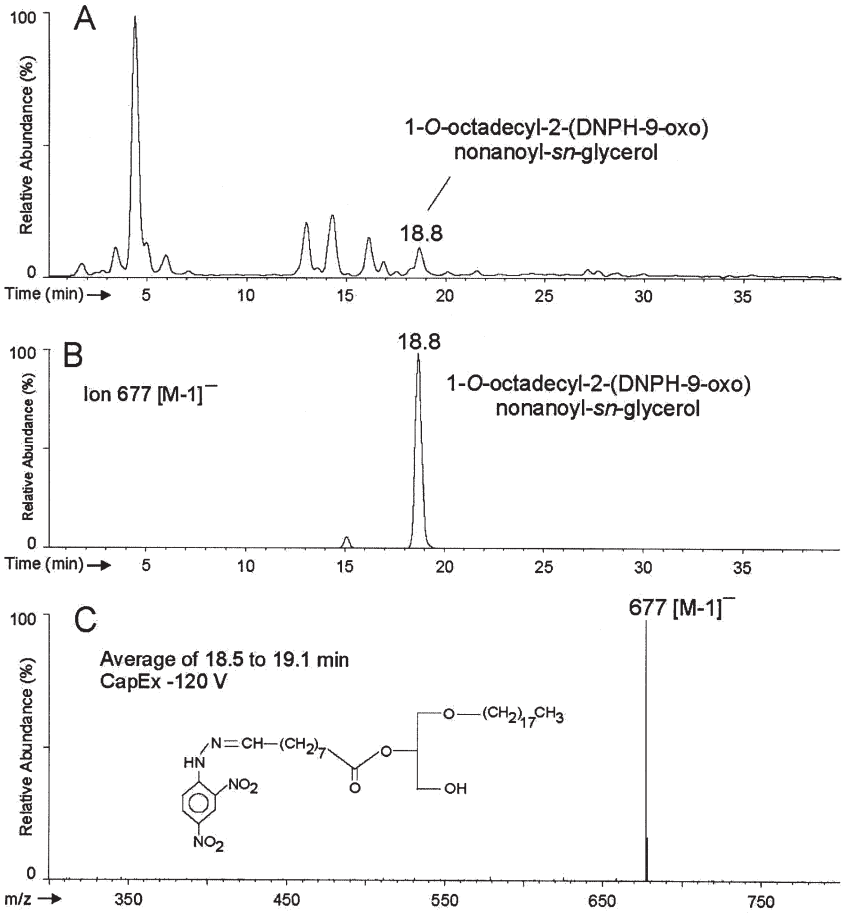
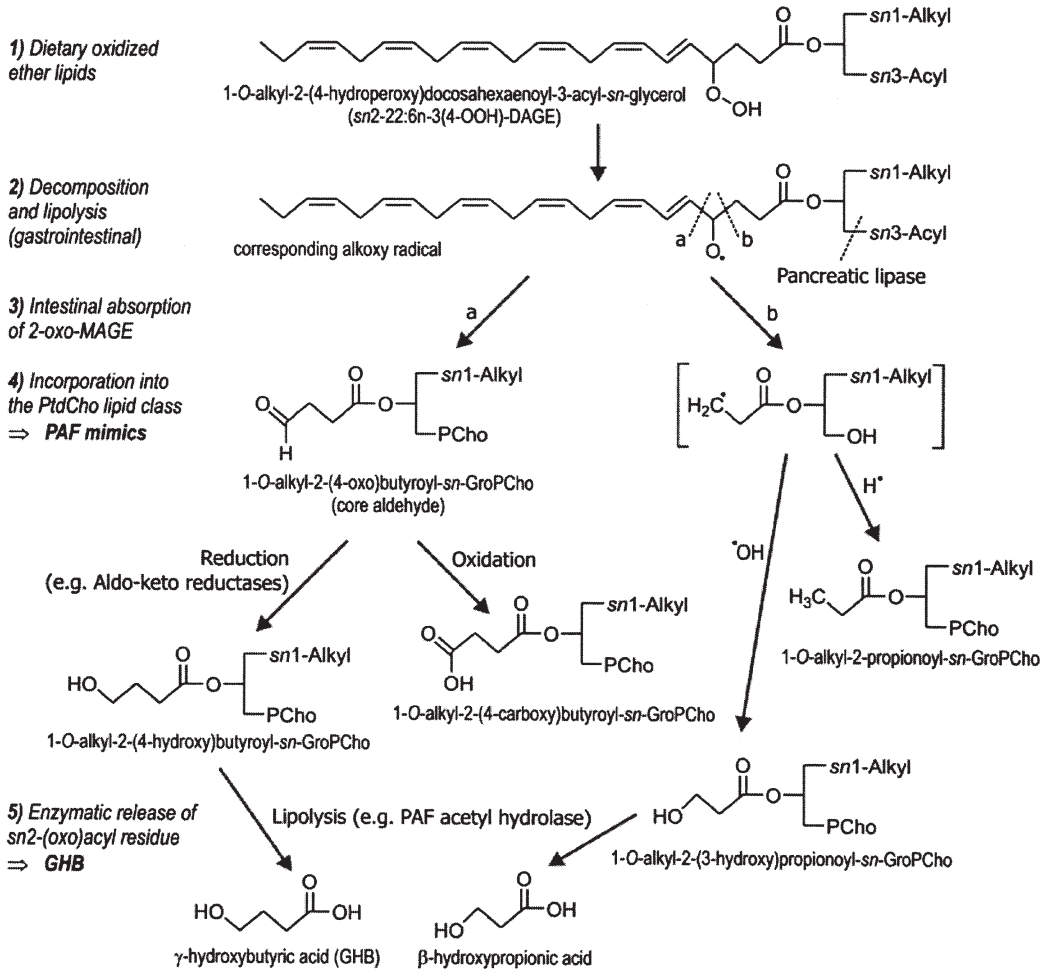


FIG. 5. Reversed-phase LC/ESI-MS analysis of autooxidized and DNPH derivatized 1-*O*-alkyl-2-acyl-*sn*-glycerols from human milk. (A) Total negative ion current profile. (B) Reconstructed single-ion chromatogram of the *m/z* 677 $[M - 1]^-$ diagnostic ion of 1-*O*-octadecyl-2-(DNPH-9-oxo)nonanoyl-*sn*-glycerol. (C) Full mass spectra averaged over the entire peak of 1-*O*-octadecyl-2-(DNPH-9-oxo)nonanoyl-*sn*-glycerol (zoomed to *m/z* 300–800) in (A).

Core aldehyde–containing triacylglycerols have been isolated from autoxidized vegetable oils (13,14) and from pig plasma lipoproteins following feeding of peroxidized fats and oils (15). Others have recovered core aldehyde–containing cholesteryl esters from autoxidized synthetic and natural (30) PUFA esters of cholesterol and from atheroma tissue (31). The present study describes the isolation and characterization of $C_{4:0}$ and $C_{5:0}$ core aldehydes from polyunsaturated diacylglycerol ethers from shark liver oil, and $C_{9:0}$ core aldehydes from human milk fat, which had not been previously reported.

Of special interest is the identification of the $C_{4:0}$ aldehyde (originating from 22:6n-3) ester in combination with the abundant 18:1n-9 alkyl moiety (50%), that is, 1-*O*-octadecenyl-2-(4-oxo)butyroyl-*sn*-glycerol, which, because of the shorter *sn*-2 chain length, could provide a higher affinity for the PAF receptor than the corresponding $C_{5:0}$ derivative, originating from 20:4n-6 esters. The 22:6n-3 (4.1% of *sn*-2 position) is highly susceptible to oxidation, and may, when esterified, yield a wide spectrum of short-chain core aldehydes with several double bonds by β-cleavage (32), such as $C_{4:0}$, $C_{7:1}$, $C_{8:2}$, and $C_{10:2}$. Another FA, highly susceptible to oxidation, is 22:5n-3 (1.6% of *sn*-2-position). This FA can yield the $C_{7:0}$ core aldehyde along with other core aldehydes with one or more double bonds. Oxidation of the remaining *sn*-2 FA would yield the $C_{9:0}$, $C_{11:0}$, $C_{12:1}$, and $C_{13:2}$ core aldehydes. We were able to characterize the *sn*-2 $C_{4:0}$, $C_{7:0}$, $C_{9:0}$, and $C_{10:1}$ short-chain aldehydes in combination with *sn*-1 18:1n-9-alkyl moiety as "free" core aldehydes.

Potential precursors of PAF mimics were also anticipated in human milk. However, we were able to identify only the 1-*O*-alkyl-2-(9-oxo)nonanoyl-*sn*-glycerol. The fatty alcohol and FA profiles of human milk preparation had previously been analyzed in our laboratory (17). The total composition was 16:0 (24%), 17:0 (2%), 18:0 (43%), and 18:1n-9 (31%) for the fatty alcohols and 16:1n-7 (3%), 18:1n-9 (36%), 18:2n-6 (5%), and 20:1n-9 (1%) for the unsaturated FA. Mild autooxidation would mainly affect the 18:2n-6 (29), and yield $C_{9:0}$ core aldehydes with minor quantities of $C_{12:1}$. Besides, oxidation of monounsaturates would yield the $C_{9:0}$ core aldehydes as well, with the exception of 20:1n-9 (1%). In other words, mild autooxida-



SCHEME 3. Proposed mechanism/pathway for formation of PAF mimics and GHB from DAGE containing docosahexaenoic acid.

tion of human milk would probably yield mainly the 1-*O*-octadecyl-2-(9-oxo)nonanoyl-*sn*-glycerol.

The isolation of the short-chain core aldehydes from the 1-*O*-alkyl-2-acyl-*sn*-glycerols is of special interest because it could lead to formation of mimics of PAF following intestinal absorption and entry into the CDP-choline pathway of PtdCho formation, bypassing the *de novo* step of diradylglycerol formation. A possible mechanism/pathway leading from a common DAGE to a PAF mimic is outlined in Scheme 3. Short-chain diacyl and diradylglycerols have been shown to be converted into short-chain PtdCho during incubation with appropriate enzymes and substrates (8). Because 1-*O*-alkylglycerols (28), and long-chain 2-*O*-alkylglycerols (28) are incorporated into glycerophospholipids following prior conversion into 1,2-diradyl-*sn*-glycerols in the intestinal microsomes, it is likely that the core aldehyde–containing 1,2-diradyl-*sn*-glycerols would also be incorporated into intestinal PtdCho to yield PAF mimics. According to Paltauf (28), the dietary 1-*O*-alkyl-*sn*-glycerols are preferentially incorporated into the glycerophosphoethanolamine *in vivo*, which, however, could be N-methylated to yield the corresponding GroPCho. Tanaka *et al.* (4) have discussed the PAF-like phospholipids formed during peroxidation of diacylglycerophosphocholines from different foodstuffs. A short-chain FA terminating in an aldehyde, hydroxyl, or carboxyl group located in the *sn*-2-position of 1-alkyl or 1-acyl glycerophosphocholine can mimic the action of natural PAF.

The short-chain core aldehydes of the *sn*-1-*O*-alkylglycerol would be expected to be readily absorbed by the intestinal mucosa because of their lower molecular weight, greater polarity, and overall similarity to monoacylglycerols. There is evidence for the intestinal uptake of intact short-chain diacylglycerols (6,7,33) as well as of oxygenated FA and acylglycerols (34–36). The presence of an alkyl group in the *sn*-1-position and a short-chain core aldehyde in the *sn*-2-position would render the molecule resistant to the endogenous lipases in the absence of isomerization, as already shown for pancreatic lipase.

The alkylglycerol core aldehydes would be expected to exhibit many of the metabolic properties of the core aldehydes of acylglycerols. Thus, these aldehydes would be expected to form Schiff bases, as demonstrated with the core aldehydes of 2-monoacylglycerols (16), and the core aldehydes of PtdCho (37) and cholesteryl esters (38). In fact, the adducts of the alkyl ether core aldehydes would be anticipated to be more resistant

to a detachment from the polypeptide and protein molecules because of the presence of the alkyl ether group. We have shown elsewhere (9) that the core aldehydes of alkyl glycerophosphocholines in atheroma induce platelet aggregation and inhibit endothelium-dependent arterial relaxation.

Furthermore, it is possible that the C_4 core aldehydes of alkylglycerols as well as of acylglycerols might be reduced by aldol-keto reductases to the corresponding C_4 core alcohols, which upon lipolysis would be released as GHB. The reduction of C_4 core aldehydes of glycerophosphocholine has already been demonstrated by Srivastava *et al.* (10), and the hydrolysis of C_4 core alcohol containing glycerophosphocholines to GHB has also been shown (4). Scheme 2 outlines a plausible mechanism/pathway for the formation of GHB from a DAGE containing a docosahexaenoic acid. Although the reduction of triacylglycerol core aldehydes to the corresponding core alcohols has not been specifically shown, such a reduction has been demonstrated for the neutral cholesteryl ester core aldehydes (Bhatnagar, 2006, personal communication). The broad substrate specificity of the aldol-keto reductases (39) promises that such reductions would take place also with triradylglycerol core aldehydes. The C_4 core alcohol groups associated with the primary positions of the triradylglycerol or triacylglycerol molecule would be anticipated to be readily released by the lingual and gastric lipases, which preferentially attack the short-chain FA (40). Any C_4 core alcohol groups associated with the secondary position of the glycerol molecule would have to undergo isomerization to a primary position before enzymatic release, as the *sn*-2-position is not known to be attacked directly.

In the brain, GHB is known to be formed from gamma-aminobutyric acid (GABA) (41) as well as from succinic semialdehyde (SSA) derived from GABA via a specific SSA reductase. The endogenous brain levels of GHB (2–5 nmol/g) are believed to be too low to stimulate $GABA_B$ receptors (K_i between 80 and 120 μM), although the synaptic cleft concentration is not known (42). Cortical GHB concentrations approaching 250 nmol/g, which are sufficient to stimulate $GABA_B$ receptors have been obtained by administration of 100–400 mg/kg of exogenous GHB (43). In any event, exogenously administered GHB causes a wide range of physiological and pharmacological properties, including addiction, tolerance, withdrawal, and intoxication, which are probably mediated via the $GABA_B$ receptor (44). This review shows that dietary sources are likely to contribute readily metabolizable precursors of GHB, the supply of which would increase with the increased consumption of omega-3 FA that is currently being actively promoted.

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Insulin-Status-Dependent Modulation of FoF_1-ATPase Activity in Rat Liver Mitochondria

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ABSTRACT: Early and late effects of alloxan diabetes and insulin treatment on mitochondrial membrane structure and function were evaluated by studying the kinetic properties of mitochondrial membrane marker enzyme FoF_1-ATPase and its modulation by membrane lipid/phospholipid composition and membrane fluidity. Under all experimental conditions the enzyme displayed three kinetically distinguishable components. In 1wk-old diabetic animals the enzyme activity was unchanged; however, K_m and V_{max} of component I increased and K_m of component II decreased. Insulin treatment resulted in lowering of K_m and V_{max} of components II and III. One-mon diabetic state resulted in decreased enzyme activity, whereas insulin treatment caused hyperstimulation. K_m of components I and II decreased together with decreased V_{max} of all the components. Insulin treatment restored the K_m and V_{max} values. In late-stage diabetes the catalytic efficiency of components I and II increased; insulin treatment had drastic adverse effect. Binding pattern of ATP was unchanged under all experimental conditions. Diabetic state resulted in progressive decrease in energy of activation in the low temperature range (E_L). Insulin treatment lowered the energy of activation in the high temperature range (E_H) without correcting the E_L values. The phase transition temperatures increased in diabetic state and were not corrected by insulin treatment. Long-term diabetes lowered the total phospholipid content and elevated the cholesterol content; insulin treatment had partial restorative effect. The membrane fluidity decreased in general in diabetic condition and was not corrected by insulin treatment at late stage. Regression analysis studies suggest that specific phospholipid classes and/or their ratios may play a role in modulation of the enzyme activity.

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Diabetes mellitus is the condition characterized by defective glucose metabolism with absolute or relative deficiencies in insulin secretion and/or in insulin action (1–4). It is also recognized that insulin, besides regulating glucose metabolism, also regulates practically all anabolic processes. These include transport of monosaccharides and amino acids, lipid synthesis, protein synthesis, turnover of RNA, and phosphorylation of several metabolic intermediates (2). It has been reported that the plasma phospholipids profile changes in Type 1 and Type 2 diabetic patients and in rat model of diabetes (5–7). It has also been reported that in human diabetics the plasma phospholipid transport protein (PLTP) decreased significantly (6–8). Changes in phospholipid composition and cholesterol content of reticulocytes and erythrocytes in human diabetics have been reported (9–11). These changes are believed to be responsible for acceleration of maturation process and/or decreased lifespan of red blood cells (11). Likewise, it has been shown that in experimental diabetes the phospholipid and FA composition of liver as well as heart microsomes changes significantly (12,13). Specifically, the activities of 18:3 and 22:6 desaturases decrease, resulting in a decrease in unsaturation index (12,14). The changes in lipid metabolism have been correlated with Na^+,K^+-ATPase activity in nerve, human placenta, and sarcolemmal membrane from rat hearts (12,14,15). A few studies have also been carried out to examine the effects of diabetes-induced disturbances in mitochondrial function and lipid metabolism (16–18).

The FoF_1-ATPase (Complex V) of mitochondria is localized in the inner membrane and requires specific phospholipids—diphosphatidyl glycerol (DPG), PC, and PE for its activity (19–21). The enzyme *in situ* utilizes transmembrane proton gradient and membrane potential $\Delta\Psi$, generated during substrate oxidation to synthesize ATP. The enzyme is a reversible proton-translocating ATPase and can generate a transmembrane proton gradient and $\Delta\Psi$, by hydrolyzing ATP (20,21). Thus, the enzyme is crucial for energy transduction functions in mitochondria (20,21). Because of its location in the inner membrane and specific requirement for phospholipids (19–21), the enzyme becomes an ideal candidate to evaluate the modulatory effects of diabetes-induced changes in mitochondrial lipid/phospholipid composition.

In view of these facts, we examined the effects of alloxan-induced diabetes at early and late stages on the kinetic properties of rat liver mitochondrial FoF_1-ATPase. Effects of insulin treatment were also evaluated. These findings are summarized in the present communication.

EXPERIMENTAL PROCEDURES

Chemicals. BSA fraction V, 1,6-diphenyl-1,3,5-hexatriene, and sodium salt of EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium salt of ATP was obtained from SRL (Mumbai). Silica gel G was from E. Merck (Darmstadt, Germany), and NPH insulin (40 U/mL) was obtained

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Abbreviations: CHL, cholesterol; DPG, diphosphatidyl glycerol; Lyso, lysophospholipid; PI, phosphatidylinositol; PLTP, plasma phospholipid transport protein; PS, phosphatidylserine; s.c., subcutaneously; SPM, sphingomylein; TPL, total phospholipid.

from Lilli (Fegersheim, France). All other chemicals were of AR grade and were purchased locally.

Animals. Adult male albino rats of Charles-Foster strain (weighing between 200 and 250 g) were used. The animals were fasted overnight, and the diabetic state was induced by injecting 12 mg alloxan/100 g bogy weight subcutaneously (s.c.) (22,23). Alloxan solutions were prepared freshly in saline. The control rats received an equivalent volume of the vehicle.

Experiments were carried out at the end of 1 wk or 1 mon of induction of diabetic state to ascertain the early onset and long-term effects, respectively (16,24). Diabetic state was confirmed in terms of hyperglycemia, polyuria, and glucosuria as detailed previously (16). For 1-wk studies the diabetic animals received insulin from the fifth day of induction of diabetes for three consecutive days, and for 1-mon studies diabetic animals received insulin starting from the fourth week of induction of diabetes for seven consecutive days at a dose of 0.8 units of NPH insulin/100 g body weight twice daily (around 7:00 AM and 6:00 PM) by s.c. route (22,23).

Isolation of mitochondria. Isolation of liver mitochondria was carried out by the procedure described earlier using the isolation medium comprising 0.25 M sucrose, 10 mM Tris-HCl buffer, pH 7.4, and 1 mM EDTA; 250 µg BSA/mL of isolation medium was included (25).

Estimation of protein was by the method of Lowry *et al.* (26) using BSA as the standard.

Analytical methods. The extraction of mitochondrial lipids/phospholipids, estimation of cholesterol, and determinations of phospholipid profile by TLC and membrane fluidity were according to the procedures described previously (27–30).

ATPase assay. Measurements of ATPase activity were carried out in the assay medium (total volume 0.1 mL) containing 50 mM Tris-HCl buffer, pH 7.4, 75 mM KCl, 0.4 mM EDTA, 6 mM $MgCl_2$, and 100 µM 2,4-dinitrophenol. After preincubating the mitochondrial protein (30–50 µg) in the assay medium at 37ºC for 1 min, the reaction was initiated by the addition of ATP at a final concentration of 5 mM (31). The reaction was terminated after 10 min by the addition of 0.1 mL of 5% (wt/vol) SDS solution, and the amount of librated inorganic phosphorus was estimated by the method of Katewa and Katyare (32). For the substrate kinetics studies, concentration of ATP was varied in the range from 0.1 mM to 10 mM. For temperature kinetics studies, experiments were carried out with fixed ATP concentration (5 mM), and the temperature was varied from 5 to 53ºC with an increment of 4ºC at each step and, depending on the temperature, the reaction was carried out for 5–10 min. Reaction velocity, v, is expressed as µmol of P_i liberated/10 min/mg protein.

Kinetics data analysis. The data for substrate kinetics were computer analyzed by three methods: the Lineweaver-Burk, Eadie-Hofstee, and Eisenthal and Cornish-Bowden plots for the determination of K_m and V_{max} using Sigma Plot version 6.1 (23,33). The values of K_m and V_{max} obtained by the three methods were in close agreement and were averaged. The K_{cat} or turnover number, which represents the number of substrate molecules transformed s^{-1}, was derived from corresponding V_{max} values (in mol) by using the equation (34):

$$K_{cat} = V_{max} \times N/(3{,}600 \times 5.60 \times 10^5) \quad [1]$$

where N is Avogadro's number and 5.60×10^5 is the molecular weight of FoF_1-ATPase (35). The ratio K_{cat}/K_m [M] is taken as the index of the catalytic efficiency of the enzyme (34). However, it is not possible to know the number of enzyme molecules/mg of mitochondrial protein, Hence we normalized the K_{cat}/K_m values to ${}^{App}K_{cat}/K_m$ by dividing the K_{cat}/K_m values by corresponding values of v at 37ºC. The ${}^{App}K_{cat}/K_m$ values thus calculated refer to unit mitochondrial protein. The data on temperature kinetics were analyzed for determination of energies of activation in the high and low temperature ranges (E_H and E_L, respectively) and phase transition temperature (T_t) according to the method described previously (23).

The regression analysis across the groups and statistical evaluation of the data by ANOVA was carried out using Jandel Sigmastat Statistical Software, version 2.0.

RESULTS

Effects of alloxan-induced diabetes and subsequent treatment with insulin on body and liver weights conformed to our previously reported observations (data not shown) (16).

As expected, the blood sugar levels increased by 3.6- and 4.6–fold, respectively, in the two diabetic groups. Insulin treatments had partial restorative effect, and the serum glucose levels were always higher compared with those in the corresponding control groups (Table 1). Other diabetes parameters such as polyuria and glucosuria were in conformity with our earlier published values (16).

In preliminary studies, we determined the effect of alloxan-induced diabetes on FoF_1- ATPase activity in the liver mitochondria at 25 and 37ºC; the data are given in Table 2. The enzyme activity was not affected in 1-wk diabetic animals. Although insulin treatment marginally increased the activity at 25ºC, the activity ratio decreased. By contrast, in 1-mon diabetic animals the activity decreased by 58%. Insulin treatment resulted in hyperstimulation (64% and 41% increase, respec-

TABLE 1
Effects of Alloxan-Induced Diabetes and Subsequent Treatment with Insulin on Blood Glucose Level

Group	Treatment	Serum glucose (mM)
One week	Control	4.21 ± 0.35
	Diabetic	15.02 ± 0.65[a]
	Diabetic + insulin	6.01 ± 0.35[a,b]
One month	Control	4.31 ± 0.33
	Diabetic	19.69 ± 0.98[a]
	Diabetic + insulin	6.63 ± 0.39[a,b]

The results are given as mean ± SEM of 12–16 independent experiments in each group.
[a]$P < 0.05$ compared with the corresponding control.
[b]$P < 0.05$ compared with the corresponding diabetic.

TABLE 2
Effect of Alloxan-Induced Diabetes and Subsequent Treatment with Insulin on FoF_1-ATPase Activity in Rat Liver Mitochondria

Group	Treatment	Activity (μmole P_i liberated/10 min/mg protein) 25°C	37°C	Activity Ratio
One week	Control	0.90 ± 0.07	2.24 ± 0.13	2.49 ± 0.10
	Diabetic	0.99 ± 0.07	2.21 ± 0.07	2.24 ± 0.12
	Diabetic + insulin	1.03 ± 0.06	2.23 ± 0.10	2.17 ± 0.10[a]
One month	Control	0.85 ± 0.07	2.10 ± 0.08	2.46 ± 0.11
	Diabetic	0.36 ± 0.03[a]	0.84 ± 0.06[a]	2.31 ± 0.10
	Diabetic + insulin	1.41 ± 0.10[a,b]	2.80 ± 0.13[a,b]	1.99 ± 0.11[a]

The results are given as mean ± SEM of 6–8 independent experiments in each group.
Activity ratio = activity at 37°C/activity at 25°C.
[a]$P < 0.05$ compared with the corresponding control.
[b]$P < 0.05$ compared with the corresponding diabetic.

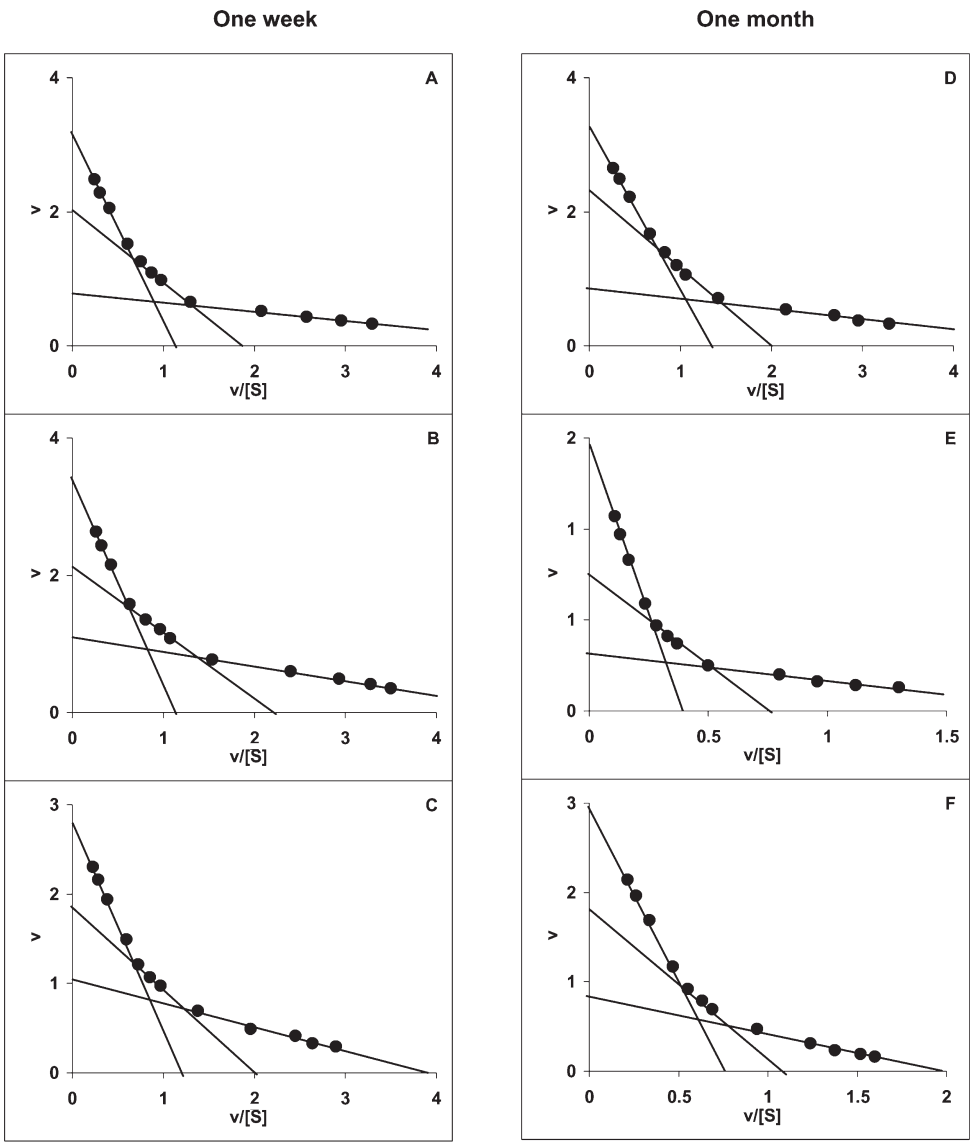

FIG. 1. Typical Eadie-Hofstee plots for FoF_1-ATPase from liver mitochondria. The enzyme activity *v* is plotted vs. *v*/[S], where *v* is the enzyme activity at the given ATP concentration [S] for (A) 1-wk control, (B) 1-wk diabetic, (C) 1-wk insulin-treated diabetic, (D) 1-mon control, (E) 1-mon diabetic, and (F) 1-mon insulin-treated diabetic. The plots are typical of 6–8 independent experiments in each group.

tively, at the two temperatures), ultimately leading to a significant and greater decrease in the activity ratio (Table 2).

In view of these differential effects of insulin treatment (Table 2), further experiments were carried out to examine the kinetics behavior of the enzyme as a response to change in the substrate, that is, ATP concentration. Analysis by three methods cited above (23,33) revealed that in the control as well as all the experimental groups the enzyme activity resolved in three kinetically distinguishable components. It has been shown earlier by us and other researchers that the rat liver mitochondrial FoF_1-ATPase activity resolves in three kinetically distinguishable components (36,37). It has been suggested earlier that the kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate (32). Typical Eadie-Hofstee plots for all groups are shown in Figure 1A–F. K_m and V_{max} values of the three components as affected by insulin status are given in Table 3. Thus, in the 1-wk diabetic group, both K_m and V_{max} of component I increased, whereas K_m of component II decreased. Insulin treatment restored the K_m and V_{max} of component I. For component II both K_m and V_{max} decreased. Long-term diabetic state had a general V_{max} lowering effect, and the K_m of component I and II decreased. Treatment with insulin restored V_{max} values in general near normality, whereas K_m of component I increased by 2-fold and that of component II was restored.

In view of the observed changes in the K_m and V_{max} values under the different experimental conditions, we computed the catalytic efficiency $^{App}Kcat / K_m$ of the three components. The data are given in Table 4. As is evident, 1-wk diabetic state did not affect the catalytic efficiency of any of the kinetic components. Treatment with insulin reduced the catalytic efficiency of component III. One-mon diabetic state increased the catalytic efficiency of components I and II. Insulin treatment, in general, reduced the catalytic efficiency of all the kinetic components; the effect being most pronounced on component I (Table 4).

Analysis of substrate kinetics data by Hill plots indicated that up to 1.15 mM ATP concentration one ATP molecule was bound; beyond this concentration of substrate two molecules of ATP were bound under all experimental conditions (data not shown).

In the next set of experiments, we evaluated the effect of alloxan-induced diabetes and subsequent treatment with insulin on the temperature-dependent change in the enzyme activity. The typical activity-vs.-temperature plots and corresponding Arrhenius plots for 1-wk and 1-mon groups are shown in Figures 2 and 3, respectively. The plots of activity vs. temperature and the corresponding Arrhenius plots for diabetic and insulin-treated diabetic animals (early as well as late stage) differed considerably from the controls. The optimum temperature in the control group (49°C) increased by 4°C to 53°C in both the diabetic groups. At the early stage treatment with insulin was ineffective in normalizing the optimum temperature, whereas at late stage the optimum temperature was restored to normality after insulin treatment (Figs. 2 and 3). The differences were also reflected in terms of the energies of activation in high and low temperature ranges (E_H and E_L, respectively) and phase transition temperature (T_t) (Table 5). Thus, in 1-wk diabetic animals the value of E_L decreased; insulin treatment lowered the value of E_H (Table 5). In the 1-mon diabetic group the pattern was repeated and the decrease in E_L was more pronounced. Insulin treatment, besides lowering the E_H, caused further decrease in E_L. The generalized feature in both the groups was increase in T_t in diabetes, which could not be corrected by insulin treatment in the 1-mon group (Table 5).

The observed changes in the substrate and temperature kinetics properties prompted us to examine the effects of insulin status on lipid/phospholipid profiles of the mitochondria. These results are given in Tables 6 and 7. In the 1-wk diabetic group the content of total phospholipid (TPL) and cholesterol (CHL) did not change significantly, and insulin treatment had no effect. Nevertheless the membrane fluidity decreased significantly, which was corrected by insulin treatment. By contrast, in the 1-mon diabetic group the TPL content decreased by 32%, and CHL content increased by 51%. This was reflected in terms of decreased molar ratio of the two entities (data not shown) and lowering of membrane fluidity. Insulin treatment restored CHL content but had marginal effect on

TABLE 3
Effect of Alloxan-Induced Diabetes and Subsequent Treatment with Insulin on Substrate Kinetics Properties of FoF_1-ATPase in Rat Liver Mitochondria

		Component I		Component II		Component III	
Group	Treatment	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
One week	Control	0.19 ± 0.01	0.88 ± 0.05	1.39 ± 0.07	2.16 ± 0.05	2.92 ± 0.16	3.24 ± 0.12
	Diabetic	0.24 ± 0.01[a]	1.03 ± 0.07	1.10 ± 0.06[a]	2.03 ± 0.07	2.92 ± 0.21	3.32 ± 0.07
	Diabetic + insulin	0.17 ± 0.01[b]	0.87 ± 0.03[a]	0.72 ± 0.07[a,b]	1.68 ± 0.06[a,b]	2.20 ± 0.16[a,b]	2.71 ± 0.08[a,b]
One month	Control	0.20 ± 0.01	0.87 ± 0.06	1.41 ± 0.11	2.13 ± 0.05	2.85 ± 0.14	3.27 ± 0.11
	Diabetic	0.12 ± 0.01[a]	0.35 ± 0.03[a]	0.77 ± 0.04[a]	0.77 ± 0.06[a]	3.27 ± 0.24	1.59 ± 0.06[a]
	Diabetic + insulin	0.39 ± 0.03[a,b]	0.97 ± 0.05[b]	1.28 ± 0.09[b]	1.87 ± 0.12[a,b]	3.07 ± 0.26	2.83 ± 0.10[a,b]

The K_m (mM) and V_{max} (μmole of Pi liberated / 10 min / mg protein) values were calculated as described in the text.
The results are given as mean ± SEM of 6-8 independent experiments in each group. As indicated in the text, the kinetic components represent the potential and the response of the enzyme to increasing concentrations of the substrate.
[a]$P < 0.05$ compared to the corresponding control.
[b]$P < 0.05$ compared to the corresponding diabetic.

TABLE 4
Effect of Alloxan-Induced Diabetes and Subsequent Treatment with Insulin on $^{App}K_{cat}/K_m$ Values for the Kinetic Components of FoF_1-ATPase in Rat Liver Mitochondria

		$^{App}K_{cat}/K_m \times 10^{11}$		
Group	Treatment	Component I	Component II	Component III
One week	Control	5.89 ± 0.28	2.30 ± 0.09	1.60 ± 0.11
	Diabetic	5.59 ± 0.31	2.52 ± 0.18	1.59 ± 0.08
	Diabetic + insulin	6.23 ± 0.48	3.36 ± 0.24	1.77 ± 0.09
One month	Control	5.92 ± 0.51	2.39 ± 0.12	1.67 ± 0.07
	Diabetic	9.06 ± 0.71[a]	3.15 ± 0.21[a]	1.89 ± 0.10
	Diabetic + insulin	3.90 ± 0.11[a,b]	1.88 ± 0.05[a,b]	1.33 ± 0.09[a,b]

The values of $^{App}K_{cat}/K_m$ were computed as described in the text. The results are given as mean ± SEM of 6–8 independent experiments in each group.
[a]$P < 0.05$ compared with the corresponding control.
[b]$P < 0.05$ compared with the corresponding diabetic.

TPL content; the membrane fluidity was not restored to normality (Table 6).

Analysis of phospholipid profile (Table 7) revealed that diabetic state had a generalized effect of increasing lysophospholipid (Lyso), sphingomylein (SPM), phosphatidylinositol (PI), and phosphatidylserine (PS), and a tendency to decrease PC and PE. Insulin treatment could correct the Lyso component and partially correct the PC and PE composition (Table 7). The computed contents of the individual phospholipid classes were generally consistent with the observed changes (data not shown but can be easily derived by multiplying the values of TPL with percent composition of individual phospholipid class).

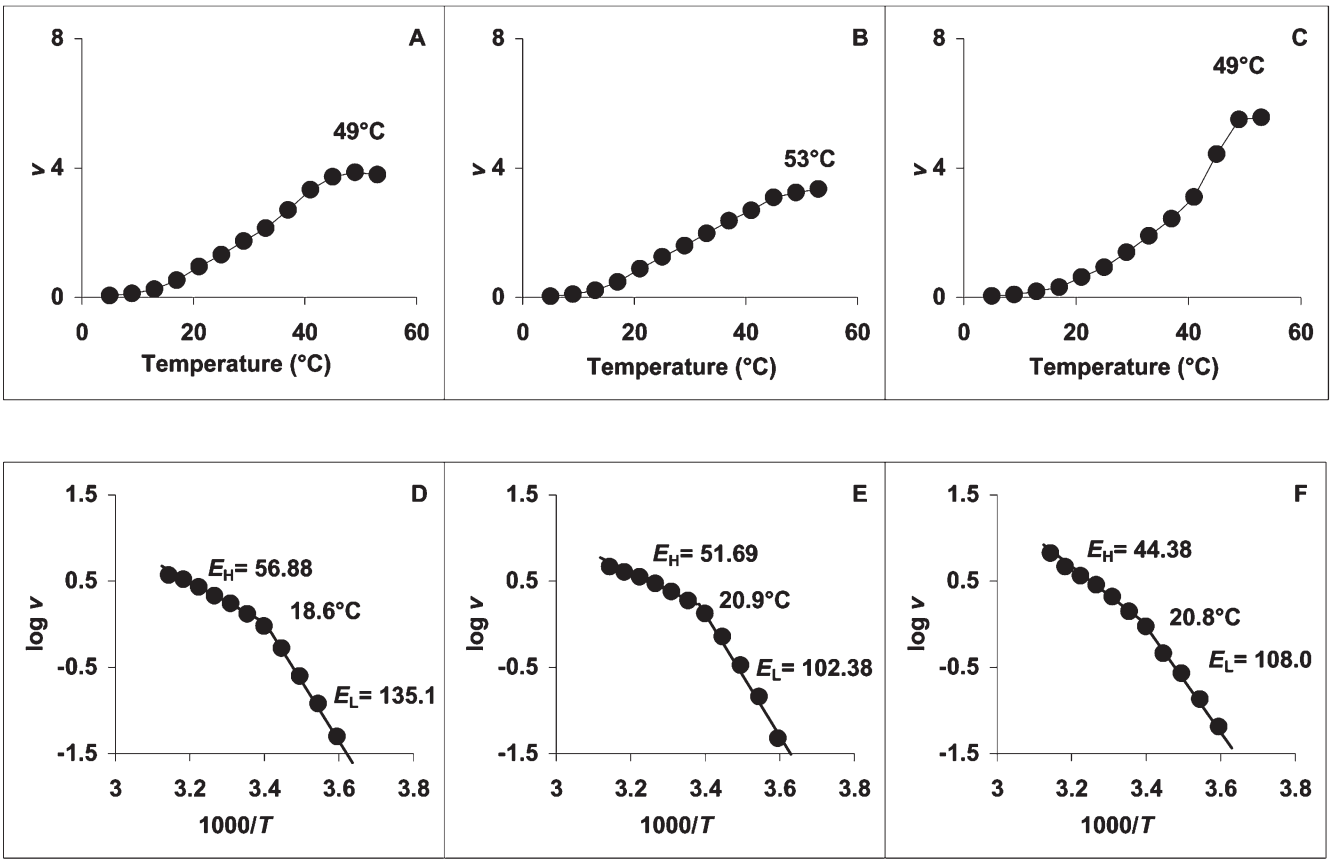

FIG. 2. Typical plots depicting dependence of enzyme activity on the temperature, and corresponding Arrhenius plots for 1-wk groups. In temperature curves, enzyme activity *v* is plotted vs. temperature (°C) for (A) Control, (B) diabetic, and (C) insulin-treated diabetic groups. In Arrhenius plots log *v* is plotted against 1,000/*T*, where *v* represents the activity at corresponding absolute temperature *T*, for (D) control, (E) diabetic, and (F) insulin-treated diabetic groups. Values of energies of activation in high and low temperature ranges (E_H and E_L) are in KJ/mole. Phase transition temperature T_t is in °C. The plots are typical of 6–8 independent experiments in each group.

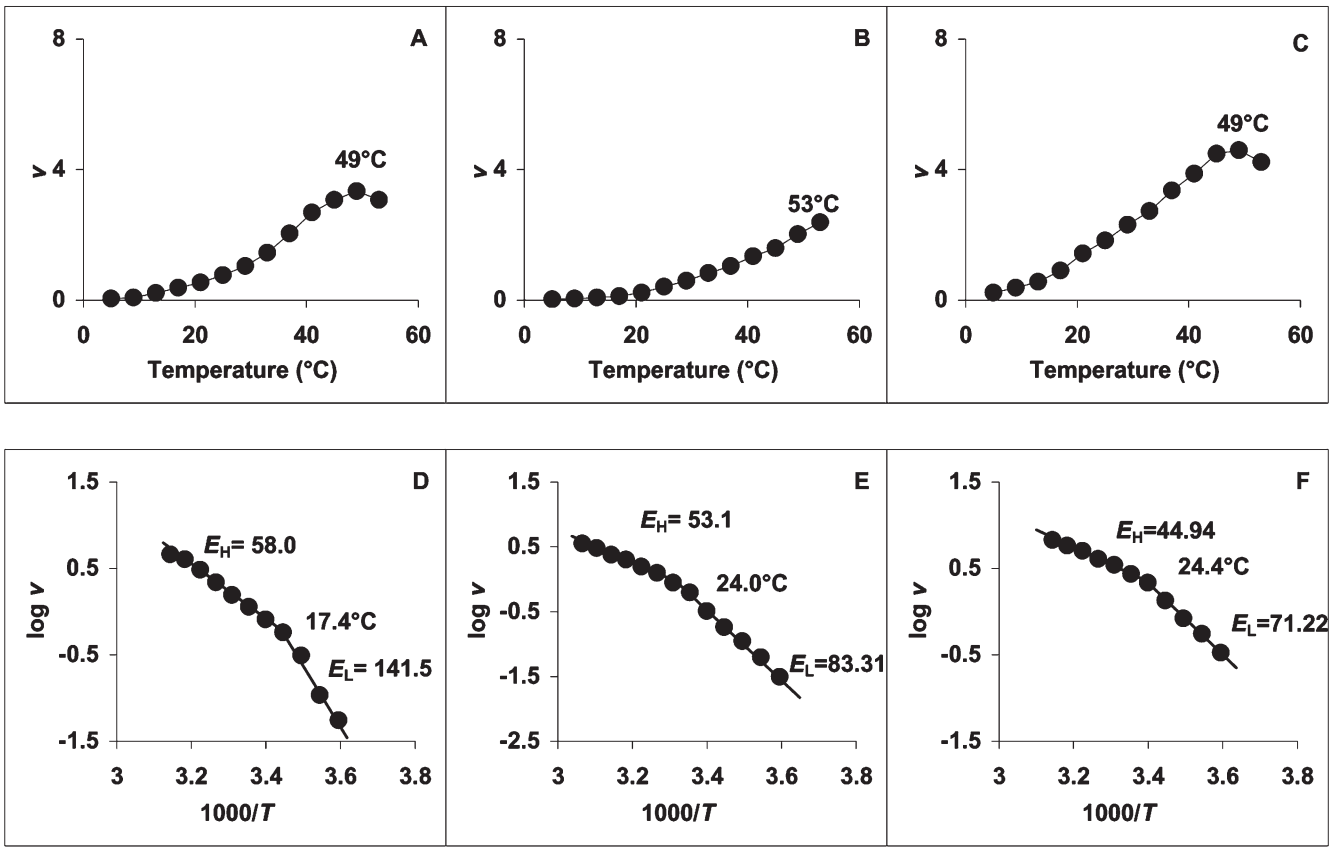

FIG. 3. Typical plots depicting dependence of enzyme activity on the temperature, and corresponding Arrhenius plots, for 1-mon groups. In temperature curves, enzyme activity *v* is plotted vs. temperature (ºC) for (A) control, (B) diabetic, and (C) insulin-treated diabetic groups. In Arrhenius plots log *v* is plotted against 1,000/*T* for (D) control, (E) diabetic, and (F) insulin-treated diabetic groups. Values of energies of activation in high and low temperature ranges (E_H and E_L) are in KJ/mole. Phase transition temperature T_t is in °C. The plots are typical of 6–8 independent experiments in each group.

DISCUSSION

As these results show, FoF_1-ATPase activity did not change in the early stage of alloxan-induced diabetes, or with subsequent treatment with insulin, whereas at the late stage the enzyme activity decreased to a great extent and insulin treatment caused hyperstimulation (Table 2). These results are consistent with our earlier findings on the oxidative phosphorylation in the liver mitochondria, which was not affected by early diabetic stage but at later stage the energy coupling efficiency decreased to a significant extent and insulin treatment more or less normalized the oxidative phosphorylation parameters (16).

The important observations of the present studies were altered substrate and temperature kinetics properties. The observation that the diabetic state resulted in lowering of K_m of component II in both the groups suggests that this is an adaptive mechanism. However, this adaptive mechanism seems to have the opposite effect on the K_m of component I. A similar situation of opposite effects was obtained even with respect

TABLE 5
Effect of Alloxan-Induced Diabetes and Subsequent Treatment with Insulin on Arrhenius Kinetics Properties of FoF_1-ATPase in Rat Liver Mitochondria

Group	Treatment	E_H	E_L	T_t
One week	Control	57.54 ± 2.46	137.2 ± 5.14	18.83 ± 0.67
	Diabetic	51.48 ± 3.33	102.0 ± 5.93[a]	21.26 ± 0.22[a]
	Diabetic + insulin	44.73 ± 2.04[a]	108.8 ± 5.31[a]	20.10 ± 0.78
One month	Control	57.20 ± 1.58	140.8 ± 4.99	17.71 ± 0.47
	Diabetic	52.80 ± 2.15	83.49 ± 5.21[a]	22.03 ± 1.35[a]
	Diabetic + insulin	45.31 ± 3.10[a]	70.62 ± 4.70[a]	23.72 ± 1.68[a]

The results are given as mean ± SEM of 6–8 independent experiments in each group.
[a]$P < 0.05$ compared with the corresponding control.

TABLE 6
Effects of Alloxan-Induced Diabetes and Subsequent Treatment with Insulin on TPL and CHL Content of Rat Liver Mitochondria

Group	Treatment	TPL (μg/mg protein)	CHL (μg/mg protein)	Fluorescence polarization (p)
One week	Control	176.2 ± 3.05	53.9 ± 1.73	0.151 ± 0.006
	Diabetic	179.4 ± 5.07	56.3 ± 2.13	0.205 ± 0.003[a]
	Diabetic + insulin	183.1 ± 7.40	52.2 ± 0.84	0.146 ± 0.002[b]
One month	Control	172.8 ± 2.47	52.8 ± 1.41	0.153 ± 0.006
	Diabetic	118.1 ± 7.58[a]	79.8 ± 1.88[a]	0.254 ± 0.005[a]
	Diabetic + insulin	138.2 ± 5.27[a,b]	47.6 ± 1.04[a,b]	0.201 ± 0.002[a,b]

The results are given as mean ± SEM of 6–8 independent experiments in each group.
[a]$P < 0.05$ compared with the corresponding control.
[b]$P < 0.05$ compared with the corresponding diabetic.

TABLE 7
Effects of Alloxan-Induced Diabetes and Subsequent Treatment with Insulin on Phospholipid Composition of Rat Liver Mitochondria

	Phospholipid Composition (% of total)					
	One week			One month		
Class	Control	Diabetic	Diabetic + insulin	Control	Diabetic	Diabetic + insulin
Lyso	1.54 ± 0.04	4.70 ± 0.30[a]	1.97 ± 0.11[a,b]	1.99 ± 0.06	4.98 ± 0.31[a]	1.18 ± 0.08[a,b]
SPM	2.82 ± 0.15	5.21 ± 0.23[a]	2.87 ± 0.12[b]	2.92 ± 0.17	4.75 ± 0.15[a]	5.25 ± 0.29[a]
PC	45.09 ± 0.41	43.67 ± 0.53[a]	45.03 ± 0.45	44.57 ± 0.61	41.41 ± 0.73[a]	42.91 ± 0.50[a]
PI	1.64 ± 0.09	2.19 ± 0.11[a]	3.16 ± 0.20[a,b]	1.98 ± 0.09	4.66 ± 0.17[a]	3.74 ± 0.22[a,b]
PS	1.65 ± 0.06	2.32 ± 0.10[a]	2.93 ± 0.17[a,b]	1.79 ± 0.08	5.53 ± 0.19[a]	2.77 ± 0.18[a,b]
PE	36.31 ± 0.39	30.91 ± 0.66[a]	31.49 ± 0.59[a]	35.11 ± 0.22	24.40 ± 0.42[a]	32.19 ± 0.47[a,b]
DPG	10.91 ± 0.13	10.92 ± 0.34	12.56 ± 0.26[a,b]	12.01 ± 0.64	14.29 ± 0.62[a]	12.02 ± 0.23[b]

The results are given as mean ± SEM of 6–8 independent experiments in each group.
[a]$P < 0.05$ compared with the corresponding control.
[b]$P < 0.05$ compared with the corresponding diabetic.

to insulin treatment. Thus, in the 1-wk diabetic group insulin treatment resulted in generalized decrease in K_m, whereas in the 1-mon diabetic group insulin treatment significantly elevated the K_m values of components I and II. Interestingly, long-term diabetes seemed to improve the catalytic efficiency of the enzyme (Table 4); insulin treatment of these animals drastically curtailed the catalytic efficiency (Table 4). Decrease in the values of E_L in diabetic state is another interesting feature, and may represent a modulatory response to improve catalytic efficiency (Table 5).

The above-mentioned observations suggest that the changes in lipid/phospholipid profile of mitochondria may be responsible for these modulatory effects. Indeed significant changes occurred in the mitochondrial TPL and CHL contents, membrane fluidity, and phospholipid composition and content. Because FoF_1-ATPase has known requirements of phospholipids, especially DPG, PC, and PE (19–21), it was of interest to find out whether the observed changes in kinetic properties across the groups could be correlated with lipid classes or their molar ratios. This possibility was evaluated by carrying out regression analysis across the groups, and the data are summarized in Table 8. As Table 8 shows, PE and total basic phospholipids (BPL), but not PC or SPM, are positive modulators of V_{max2} and V_{max3}. By contrast, the acidic phospholipids PI, PS, and DPG are negative modulators (Table 8). The molar ratio of TPL:CHL correlated positively with V_{max} values of all three kinetic components. V_{max} values of components II and III correlated positively with molar ratio of TPL:PI, TPL:PS, and TPL:PI + PS. A negative correlation of PC/PE, PI/BPL, PS/BPL, and DPG/BPL with V_{max2} and V_{max3} was another interesting feature. E_L correlated positively with PE, TPL/PI, TPL/PS, and TPL/PS + PI, whereas negative correlations were obtained with SPM, PI, PS, SPM/PC, PI/BPL, and PS/BPL (Table 8). Based on these observations, it may be suggested that besides changes in the phospholipid composition, the charge distribution across the membrane and especially in the lipid microdomains in which FoF_1-ATPase is embedded and changes in membrane fluidity may play a significant modulatory role.

It has been recognized that the 18:3 and 22:6 desaturase activities decrease in diabetes thus lowering the unsaturation index (12,14), The increase in T_t that we note here (Table 5) is consistent with these observations.

Mutations in mitochondrial DNA leading to dysfunction of FoF_1-ATPase complex has been proposed to be an underlying cause of mitochondrial diabetes. However dysfunction of Pyruvate dehydrogenase complex can also lead to a diabetic state (38–40). It is not clear at this stage whether a similar situation would prevail following exposure to alloxan. However, from the data presented it is apparent that changes

TABLE 8
Correlation Between Kinetics Parameters of Liver FoF_1-ATPase and Membrane Lipid/Phospholipid Composition

	Correlation with			
	Phospholipid class		Phospholipid molar ratio	
Parameter	Positive	Negative	Positive	Negative
V_{max1}	—	—	TPL/CHL (+0.644)	—
V_{max2}	PE (+0.764) BPL (+0.729)	PI (–0.660) PS (–0.734) DPG (–0.624)	TPL/CHL (+0.719) TPL/PI (+0.611) TPL/PS (+0.623) TPL/PS + PI (+0.617)	PC/PE (–0.706) PI/BPL (–0.710) PS/BPL (–0.734) DPG/BPL (–0.731)
V_{max3}	PE (+0.723) BPL (+0.822)	PI (–0.756) PS (–0.818) DPG (–0.701)	TPL/CHL (+0.712) TPL/PI (+0.714) TPL/PS (+0.731) TPL/PS + PI (+0.728)	PC/PE (–0.728) PI/BPL (–0.813) PS/BPL (–0.828) DPG/BPL (–0.767)
E_L	PE (+0.664)	SPM (–0.735) PI (–0.792) PS (–0.656)	TPL/PI (+0.796) TPL/PS (+0.796) TPL/PS + PI (+0.807)	SPM/PC (–0.730) PI/BPL (–0.773) PS/BPL (–0.779)

Values given in the parentheses indicate *r*, the regression coefficient, based on 6–8 independent experiments in each group.

in membrane lipid/phospholipid domains may play a regulatory role.

Our results also show that the aberrations introduced by alloxan-induced diabetes could not be fully corrected by treatment with insulin. This is consistent with the well-acknowledged fact that insulin does not correct all the maladies of diabetes (1,41,42). One therefore wonders whether complete restoration to normality could be achieved by combined treatment with insulin and c-peptide (41,42). This interesting possibility is worth exploring further.

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Gene Expression of Stearoyl-ACP Desaturase and Δ12 Fatty Acid Desaturase 2 Is Modulated During Seed Development of Flax (*Linum usitatissimum*)

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ABSTRACT: Flax's recent popularity in human and animal foods is mostly due to its desirable FA composition. Flax is an excellent source of omega-3 FA, which have been shown to have many health benefits. To date, little is known about the genetic and environmental factors that control the FA composition of flax seeds. To elucidate some of the important genetic components, reverse transcriptase (RT)–PCR and real-time PCR were used to determine the expression profiles of two key FA biosynthetic genes during seed development. Plants of flax cultivar AC McDuff were grown under field conditions, and RNA was extracted from ovaries and developing bolls collected from 2 d after anthesis (DAA) to maturity. Desaturation enzymes stearoyl-ACP desaturase (SAD) and Δ12 FA desaturase 2 (FAD2) were both expressed in ovaries, and their expression was differentially modulated throughout seed development. SAD was most highly expressed in ovaries. Its expression quickly decreased until 4 DAA; this was followed by a slight peak at 8 DAA, only to return to relatively low levels of expression in maturing bolls, ranging from 2.1% to 4.5% relative to the level observed in ovaries. FAD2 expression displayed a different temporal pattern. While expression of FAD2 did decrease in the early stages of seed development, expression increased starting at 8 DAA, peaking at 16 DAA, when it was 158% relative to the level observed in ovaries. FAD2, which desaturates oleic acid (18:1*cis*Δ9) into linoleic acid (18:2*cis*Δ9,12), is therefore controlled at the transcription level. To relate enzyme expression with FA profile, GC was performed on the same subsamples used for RT-PCR and real-time PCR, and proportions of palmitic, stearic, oleic, linoleic, and linolenic acids were determined for the same developmental stages. Although FAD2 expression increased from 8 to 16 DAA, relative changes in linoleic acid (18:2*cis* Δ9,12) were not observed. However, linolenic acid (ALA; α-18:3; 18:3*cis*Δ9,12,15) levels increased steadily, meaning that linoleic acid (18:2*cis*Δ9,12) is a transient substrate converted by FAD3 as quickly as it is produced by FAD2. Phenotypes are the result of genotypes, environment, and the interaction of the two. To evaluate the environmental impact on the production of FA in flax, FA profiles were assessed in a total of four environments (two locations, two years). Warm and dry environmental conditions resulted in lower levels of PUFA 18:2*cis*Δ9,12 and 18:3*cis*Δ9,12,15, and higher levels of 18:1*cis*Δ9. FAD2 expression and/or activity may therefore be affected by the environment.

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Omega-6 FA such as linoleic acid (18:2*cis*Δ9,12) and omega-3 FA such as linolenic acid (18:3*cis*Δ9,12,15) are EFA. They are components of cell membranes and play many important metabolic roles. They are precursors to long-chain FA critical to normal development (1). Adequate intake and proper balance of EFA are necessary to ensure proper physiological functions of the human body.

Numerous health benefits have been associated with functional FA, particularly omega-3 (2). Their role in reducing the risk of sudden cardiac death and coronary heart disease, cancer, and other human health risk factors has been scientifically demonstrated (3,4). Flax seed is an excellent source of α-linolenic acid (ALA), and the effects of dietary flax seed in reducing serum cholesterol and cardiovascular incidence have recently been demonstrated in model organisms such as rats and rabbits (5,6).

Omega-3 FA are found in fish, algae, and plants such as flax, walnuts, soybean, and canola. Flax seeds and flax oil are the richest sources of ALA in the North American diet. Traditional linseed varieties have approximately 45–50% ALA, whereas high-linolenic lines may comprise more than 70% ALA. Flax is therefore an oilseed of choice when it comes to designer oil because of the intrinsic quality of its oil and its genetic variability for oil composition with a potential for very high content of ALA. Solin varieties such as Agricore United Linola™ varieties have been developed for very specific markets and have less than 5% ALA.

From a plant perspective, FA are essential for cell biology not only as cell membrane structural components but also as storage lipids (7). FA biosynthesis in oilseeds is catalyzed by a set of condensing and desaturation enzymes located in plastids and endoplasmic reticulum (ER), and the series of reactions necessary for *de novo* synthesis of FA up to 18 carbons in length have been described (8,9). Of the plastid *de novo*–synthesized acyl-ACP (16:0, 18:0, and 18:1*cis*Δ9) pools in *Arabidopsis* mesophyl cells, 38% remain in chloroplasts, and 62% are cleaved from ACP by acyl-ACP thioesterases (10). The free FA are then converted to acyl-CoA by an outer-envelope long-

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Abbreviations: DAA, days after anthesis; FAD2, Δ12 FA desaturase 2; RT, reverse transcriptase; SAD, stearoyl-ACP desaturase.

chain acyl CoA synthetase (LACS) isoform, exported to ER, and used in several metabolic pathways including those of storage lipid synthesis in developing seeds, those of cutin and suberin production, and those of cuticular wax layer components synthesis, depending on tissue type and developmental stage (10–12). Desaturation of 18:0 FA into 18:1*cis*Δ9, 18:2*cis*Δ9,12 and 18:3*cis*Δ9,12,15 is performed by SAD, FAD2, and FAD3 enzymes, for which some gene members have recently been cloned in flax (13–15). Little is known about the genetic control of these enzymes, particularly whether their activity is controlled at the transcription, translation, or post-translation level.

FA composition in the seeds of oilseed crops is influenced by the environment. The phenotypic plasticity and the diversity of metabolites are the abilities of a genotype to produce multiple phenotypes and/or metabolite profiles in response to different environments. Understanding the molecular basis of this plasticity is important for breeding programs (16–18). To date, several studies have examined the role of temperature on FA biosynthetic gene expression (19,20). In soybean (*Glycine max* L.), the vital role of temperature, location, and year in FA biosynthesis has been reported (21). Soybean plants exposed to high daily temperatures were shown to produce reduced linoleic and linolenic acid and increased oleic acid content (22,23). It has also been reported that lines with reduced linolenic acid were less sensitive to wide temperature changes (24). The effect of temperature on oil composition during seed maturation has been investigated in low-linolenic flax genotypes, where high temperatures considerably decreased the relative proportions of linoleic and linolenic acids (25). In short, genotype (G), environment (E), and genotype–environment interaction (G–E) have all been demonstrated to influence plant oil production and the oil composition in mature seeds.

The objectives of the present research were threefold: to quantify the FA composition of flax during seed development; to quantify the expression levels of the genes encoding two desaturases (SAD and FAD2) in order to establish the role of transcriptional control in FA composition; and to estimate the environmental effect on FA composition.

EXPERIMENTAL PROCEDURES

Plant materials. Flax plants (*Linum usitatissimum* L. cv AC McDuff) were grown at Winnipeg (49°54' N, 97°14' W) and Morden (49°12' N, 98°61' W), Manitoba, Canada, under rainfed conditions over the growing seasons 2002 and 2003. AC McDuff is late-maturing oilseed flax with high oil content (47%), of which 50% is linolenic acid (26). At anthesis, referred to as 0 d after anthesis (DAA), individual flowers were tagged sequentially, and developing bolls were harvested at 0, 2, 4, 8, 12, 16, 20, 24, 28, and 32 DAA and at maturity. The 0 DAA samples consisted of ovaries free of other flower tissues. Sampling took place in late July and August. Developing bolls were immediately frozen in liquid nitrogen and then stored at –80°C. Subsamples were used for RNA extraction and FA profiling.

RNA isolation. Total RNA was isolated from 0 to 32 DAA samples of developing bolls and mature bolls collected in Winnipeg in summer 2002 using a Trizol procedure adapted for seed material (http://plantsciences.montana.edu/wheat-transformation/molecular.htm#totalrnamini). Briefly, 0.5 mL of extraction buffer (50 mM Tris pH 9.0, 200 mM NaCl, 1% Sarkosyl, 20 mM EDTA pH 8.0 and 5 mM DTT) was added to 50–100 mg of tissue ground in liquid nitrogen and homogenized by vortexing. Extraction was performed with 0.5 mL phenol/chloroform/isoamyl alcohol (49:49:2). After centrifugation at 18,300*g* for 5 min at 4°C, 1 mL of Trizol reagent (Invitrogen, Burlington, Ontario, Canada) was added to 0.5 mL upper aqueous phase in a fresh 2-mL tube and vortexed. Then, 0.2 mL chloroform was added, and samples were vortexed and then centrifuged at 18,300*g* for 5 min at 4°C. A total of 0.5 mL chloroform was added to 0.75 mL upper aqueous phase in a new 2-mL tube, vortexed, and centrifuged again. Approximately 0.6 mL of supernatant was precipitated by adding 1.2 mL of 100% ethanol and 90 μL of 2 M sodium acetate. The samples were then vortexed and incubated at –80°C for 1 h. Samples were centrifuged at 18,300*g* for 20 min at 4°C. The pellets were washed with 70% ethanol, air dried for about 10 min, and subsequently resuspended in RNAse-free water. RNA was quantified by spectrophotometry, and quality was verified by formaldehyde-agarose gel eletrophoresis.

First-strand synthesis, RT-PCR, and real-time PCR of SAD and FAD2. Total RNA (2 μg) from bolls collected at different developmental stages was first treated with RNase-free DNAse I (Invitrogen) and used as template in first-strand synthesis following the manufacturer's recommendations. Samples were subsequently treated with RNAse H (Invitrogen). For PCR reactions, cDNA samples were diluted 10-fold, and 1 μL was used as template. For real-time PCR reactions, first-strand cDNA samples were quantified by spectrophotometry and diluted to 200 ng/μL. Primers used in this study were designed based on FA biosynthetic gene sequences (CD760586 for linSAD and CD760583 for linFAD2, reported in Fofana *et al.* (14)) to amplify fragments of approximately 80–100 bp (Table 1). Preliminary RT-PCR reactions were performed to determine the optimized annealing temperature of each primer pair. Amplification reactions contained 1 μL of 10-fold-diluted first-strand cDNA reaction, 1X reaction buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM of each primer, 5U Taq DNA polymerase, and were completed to 50 μL with sterile RNAse-free, DNase-free water. Samples were first denatured at 94°C for 2 min, and then followed 35 cycles consisting of 94°C for 30 s; 48°C (linSAD) or 55°C (linFAD2) for 30 s, depending on primer annealing temperature; and then 72°C for 60 s. Final extension was conducted at 72°C for 10 min. RT-PCR was performed using RNA from 0-DAA to 32-DAA developing bolls and mature bolls. Aliquots of 10 μL of the PCR products were separated on 1% (wt/vol) agarose gels stained with ethidium bromide.

Real-time PCR reactions were performed in triplicate using the ABI SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California) and analyzed using an iCycler iQ real-time detection system (BioRad, Mississauga, Ontario, Canada),

TABLE 1
Primer Sequences Used for SAD and FAD2 Gene Expression Analyses by RT-PCR and Quantitative Real-Time PCR

Gene ID (GenBank) Accession number)	Forward Primer Sequence	Reverse Primer Sequence	Annealing temperature (°C) RT-PCR	Annealing temperature (°C) Real-Time PCR
linSAD (CD760586)	CGGTGACCTTCTCAACAA	AGAGCCGATGAGGTACTGA	48	48
linFAD2 (CD760583)	CATTCGTCCCTCCTTGTT	CTCATCACGATCAAGCGA	55	59

according to the manufacturer's instructions. Amplification reactions consisted of 1X SYBR Green Master Mix, 12.5 pmoles of each primer, 200 ng of first-strand synthesis cDNA obtained from developing bolls (0 to 28 DAA) as described earlier, and completed to 25 μL with sterile H_2O. Real-time PCR reactions were performed as follows: a denaturation step at 95°C for 10 min followed by 40 cycles consisting of 95°C for 30 s, 48°C (linSAD) or 59°C (linFAD2) for 30 s, and 72°C for 30 s. Following the final amplification cycle, a melting dissociation curve was generated to ensure specificity of the primers and to confirm that only a single product was present. The reactions were held for 10 s at the primer's respective annealing temperature and then ramped up by 0.5°C every 10 s for 85 steps. Three independent reactions, each with a different cDNA sample, were carried out. Means of triplicate reactions were expressed as relative gene expression at different time points compared with the expression detected at 0 DAA.

FA profiling. FAME were extracted from five bolls per replicate (three replicates in total) for each time point and for each location according to the procedure described by Fofana *et al.* (14). FA composition of developing bolls was performed using GC analysis. The weight percentage of FA was calculated according to the procedure outlined in AOAC method 996.06. ANOVA was performed using the SAS program (SAS Insttitute Inc., Cary, NC, release 8.2, 1996). The mean squares for the ANOVA were calculated by PROC GLM, and the estimates of the variance components were calculated using PROC MIXED. The mean percentages of FA content obtained from six measurements (three samples in duplicate) for each developmental stage, location, and year were presented with their standard error. FA profiles were interpreted in close relationship with weather data obtained from meteorological services for each growing season.

RESULTS

FA biosynthetic gene expression. Expression of two FA biosynthetic genes, namely SAD and FAD2, was estimated by RT-PCR and real-time PCR in flax ovaries and developing bolls from early stages to maturity. RT-PCR is considered a semiquantitative evaluation of transcript accumulation, whereas real-time PCR is a sensitive and robust tool for monitoring gene expression and providing quantitative evaluation of transcript copies (27). The accuracy of real-time PCR depends on the development of primers highly specific to the target gene. Tight single-peak melting point dissociation curves indicative of the uniqueness of the target sequence were observed for both SAD and FAD2 real-time PCR reactions. The linear relationship between threshold cycle (Ct) and cDNA templates was established. High correlation coefficient and high PCR efficiency were observed for each gene, permitting accurate quantification of transcript copy number.

Expression of linSAD and linFAD2 was measured as relative transcript abundance compared with the levels measured in ovaries (0 DAA) (28). LinSAD and linFAD2 transcripts were both highly accumulated at 0 DAA (Fig. 1A, 2A). LinSAD mRNA accumulation declined gradually between 0 and 4 DAA. A slight peak at 8 DAA was followed for the later seed developmental stages by levels of mRNA transcripts representing less than 5% of the level in ovaries (Fig. 1A). LinFAD2 transcripts were undetectable at 2 DAA. Its expression was relatively low at 4 and 8 DAA, and steadily increased to peak at 16 DAA (Fig. 2A), when the expression was nearly 1.6 times the level observed in ovaries. A slow decline was observed from 16 to 28 DAA, when expression levels were similar to that observed at 4 DAA.

RT-PCR was performed on the same RNA samples used for real-time PCR. Amplification of both genes under study using RT-PCR revealed single bands (Fig. 1B, 2B), confirming primer specificity as shown in real-time PCR. The semiquantitative RT-PCR amplification results, specifically amplicon relative intensity, closely matched the real-time PCR results for both SAD (Fig. 1A, 1B) and FAD2 (Fig. 2A, 2B) genes. RT-PCR was performed on 32-DAA and mature boll samples and showed low level of expression for SAD. No transcripts were amplified for FAD2 at these late developmental stages.

FA accumulation during seed development. Determination of FA profiles by GC analysis revealed a significant difference ($P < 0.0001$) in FA contents according to boll developmental stages. Palmitate (16:0), linoleate (18:2*cis*Δ9,12), and linolenate (18:3*cis*Δ9,12,15) were the most abundant FA at early stages, that is, 0–4 DAA (Fig. 3). The relative proportion of palmitate decreased during later seed development from 4 to 32 DAA. Meanwhile, unsaturated FA (oleate, linoleate, and linolenate) represented an increasingly large proportion of the total FA content. Omega-3 ALA (18:3*cis*Δ9,12,15) was the

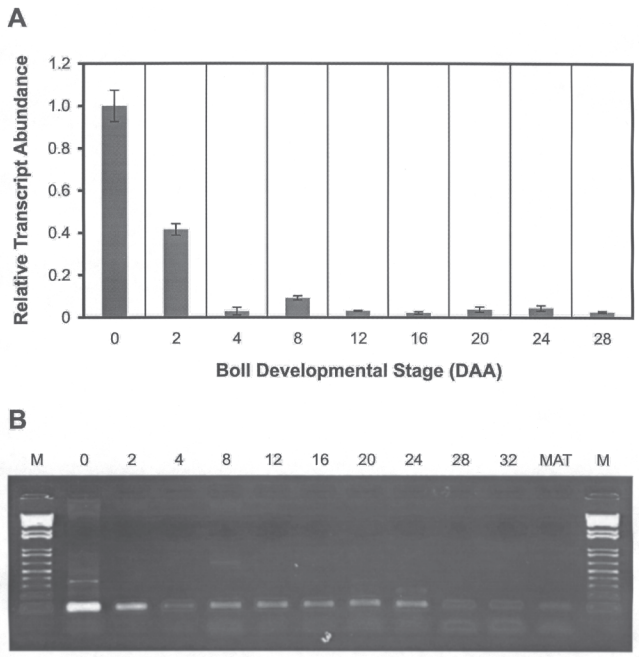

FIG. 1. Relative expression of linSAD at different developmental stages including ovaries (0 DAA) and stages of boll development ranging from 2 DAA to maturity (MAT). (A) Real-time PCR determination of the relative expression calibrated against the expression measured in ovaries (0 DAA) for developing bolls from 2 to 28 DAA. Time point expressions were obtained from triplicate independent reactions and standard errors of the ratios of the means are represented; (B) RT-PCR displayed a unique fragment whose intensity varied according to the developmental stages tested (ovaries to mature bolls) and that correlated with the quantitative real-time PCR measurements in (A). Marker (M) is 0.24–9.5 Kb RNA ladder (InVitrogen).

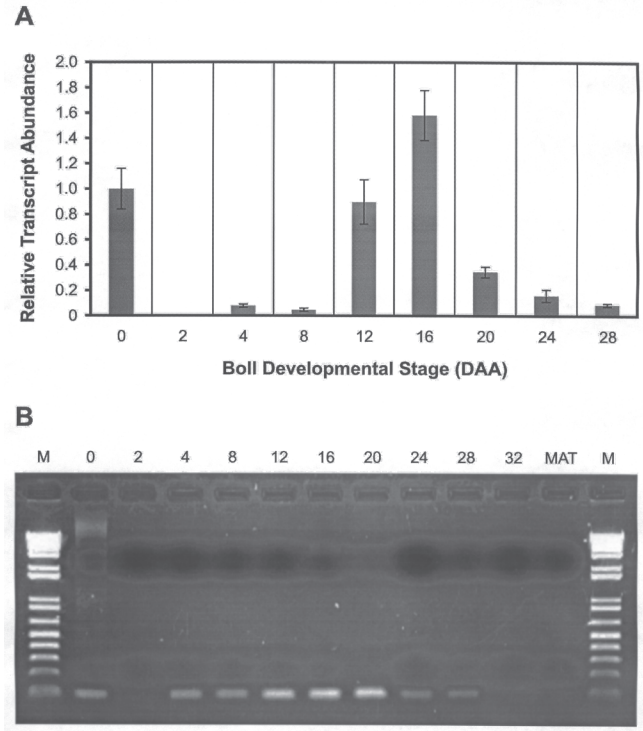

FIG. 2. Relative expression of linFAD2 at different developmental stages including ovaries (0 DAA) and stages of boll development ranging from 2 DAA to maturity (MAT). (A) Real-time PCR determination of the relative expression calibrated against the expression measured in ovaries (0 DAA) for developing bolls from 2 to 28 DAA. Time point expressions were obtained from triplicate independent reactions and standard errors of the ratios of the means are represented; Peak expression is observed at 16 DAA, when the level was nearly 1.6-fold the level observed in ovaries. (B) RT-PCR displayed a unique fragment whose intensity varied according to the developmental stages tested and that correlated with the quantitative real-time PCR measurements in (A). Marker (M) is 0.24–9.5 Kb RNA ladder (InVitrogen).

most abundant FA found from 8 to 32 DAA, exceeding 50% of the FA composition at maturity.

Environmental effect on FA accumulation. Environmental conditions are crucial during seed development. On the Canadian Prairies, flax plants bloom and the bolls mature in late July and August. Mid to late July 2002 was slightly warmer on average (22.1°C) than mid to late July 2003 (21.2°C), and the first half of August 2002 was definitely cooler (18°C) than August 2003 (23.1°C). The rainfall recorded from mid-July to mid-August was higher in 2002 (84 mm) than in 2003 (42 mm). The rainfall recorded in 2002 was similar in both locations (203 mm in Winnipeg and 205 mm in Morden). However, 2003 was drier than 2002, and Morden received only half of the rainfall recorded in Winnipeg (64 mm vs. 130 mm).

FA profiles for the same growing season were comparable at both locations (Figures 3A-C and B-D). A significant difference ($P < 0.0001$) was observed between years in each location for oleic (18:1*cis*Δ9) and linoleic (18:2*cis*Δ9,12) acids. In 2002, no difference could be observed between oleic (18:1*cis*Δ9) and linoleic acids (18:2*cis*Δ9,12) from 8 DAA to maturity within or between locations (Fig. 3A, C). In 2003 (warmer August), however, the level of 18:1*cis*Δ9 was higher than that of 18:2*cis*Δ9,12 at both locations (Fig. 3B, D). The proportion of linolenic acid (18:3*cis*Δ9,12,15) increased markedly between 8 and 20 DAA at Morden, and this trend continued up to 24 DAA at Winnipeg. The linolenic acid content accounted for the highest proportion of PUFA in both locations. Moreover, the overall 18:3*cis*Δ9,12,15 content was approximately 3–5% higher in 2002 (cool) as compared with 2003 (warm) in both locations from 12 DAA to maturity.

DISCUSSION

FA metabolism is an important biological function for living organisms, and plant oil metabolic engineering has become an attractive research target (9,29,30). In this study, we examined the expression of two FA biosynthetic genes in developing bolls of flax cv AC McDuff using semiquantitative RT-PCR and quantitative real-time PCR. FA profiles of this oilseed crop were also studied during two growing seasons, at two locations, and for a number of development stages ranging from 0 to maturity. Real-time PCR is a sensitive and robust tool for monitoring gene expression (27). Using this technique, our results

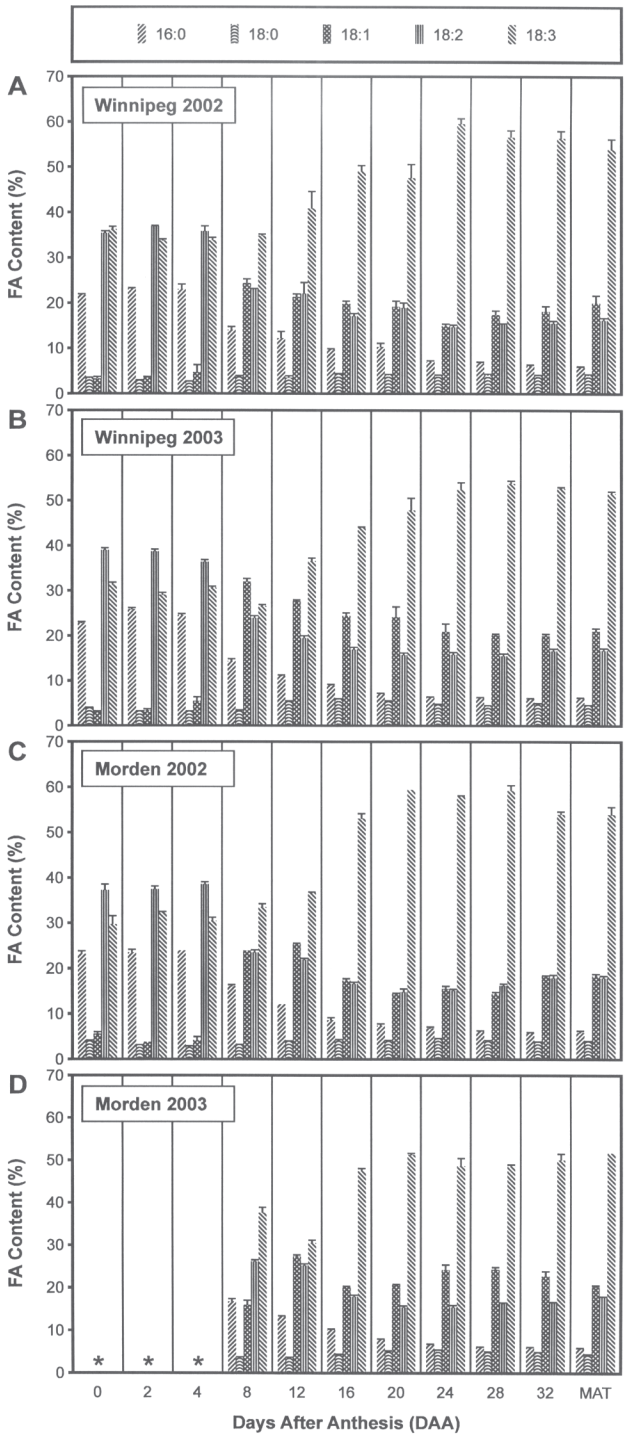

FIG. 3. FA profile obtained by GC analysis and expressed as the partitioning (%) in palmitic, stearic, oleic, linoleic, and linolenic acid measured in ovaries (0 DAA) and several stages of developing bolls ranging from 2 to 32 DAA and at maturity. The samples were obtained from two locations and during two growing seasons, namely (A) Winnipeg 2002, (B) Winnipeg 2003, (C) Morden 2002, and (D) Morden 2003. Vertical bars represent standard error of the mean obtained from six independant measurements using five bolls per replicate and run in duplicate. Missing data (*) and mature boll stage (MAT) are indicated on the *x*-axis.

showed that desaturases, also expressed early, appeared to be regulated and coordinated during boll development.

SAD expression decreased gradually from anthesis to 4 DAA, spiked at 8 DAA, and remained relatively constant in later stages of development. In flax, previous studies based on Southern hybridization have reported a single copy for SAD (13,14), although cross hybridization between closely related isoforms cannot be ruled out. Indeed, two SAD, SAD1 and SAD2, have been reported (31). However, because gene-specific primers were designed from the most seed-specific SAD gene (SAD1, AJ006957), and a single fragment was amplified, as confirmed by RT-PCR and melting curve, one might reasonably assume that the seed-specific SAD1 isoform was the one investigated in this study. The temporal regulation of this enzyme was also shown in *Brassica napus,* and the seed tissue specificity of the *Brassica* SAD was demonstrated in transgenic tobacco (32,33). To our knowledge, there are no previous reports on the expression profiling of SAD in developing flax bolls. SAD1 and SAD2 gene expressions were not studied at very early stages by Jain *et al.* (31). SAD expression does not seem to be limited to the developing seeds. Expression in ovaries (14) and in tapetum and pollen grains (33) was reported in flax and rapeseed, respectively.

The FAD2 gene encodes the enzyme that catalyzes the desaturation of oleate to linoleate (34). Two closely related FAD2 copies, as determined by Southern and cDNA sequence analyses, were found in flax cv AC Mcduff (14). FAD2 orthologs have also been cloned and characterized in a number of plant species such as rapeseed, soybean, cotton, flax, olive, maize, peanut, *Arabidopsis*, and *Lesquerella* (14,34–40), Of particular interest, two seed-specific microsomal FAD2 isoforms have been cloned (37,39,40), only one (FAD2-1) was strongly expressed in seed, whereas FAD2-2 was constitutively expressed (40). The FAD2 expression data reported in this study is consistent with that of the seed-specific FAD2-1 previously reported (40) and suggest that FAD2-1 was the isoform investigated in this study, although submembers (FAD2-1A and FAD2-1B) for this gene have been recently reported (40). However, our data could not assign which of the FAD2-1 submembers (FAD2-1A or FAD2-1B) was measured in this study. Nevertheless, this does not affect the conclusion drawn from the FAD2 expression profile reported herein. In soybean, post-transcription modification such as phosphorylation was proposed as an important mechanism for FAD2-1 activity in seed (40). This result was not contrasted with potential regulation at the transcription level. In flax, the present study demonstrated the transcriptional control of FAD2 expression during seed development. FAD2 transcript expression differed significantly from the SAD expression pattern. FAD2 expression increased sharply up to 16 DAA to a level surpassing that at anthesis and slowly decreased as the bolls matured, corroborating findings in *Arabidopsis* by Ruuska *et al.* (41), in which a bell-shaped pattern was also observed. These results, based on the whole seed-specific FAD2 gene expression, were supported by phenotypic data showing a high desaturation ratio of stearate (18:0)

across all development stages, and thereafter of oleate (18:1*cis*Δ9) and linoleate (18:2*cis*Δ9,12). Although bolls from 0 to 32 DAA were used as starting materials, our expression data from 4 DAA to maturity was well correlated with FAD2 tissue specificity as previously reported (41). Phenotypic data indicated high desaturation activities from 8 to 24 DAA followed by a plateau in accumulation of oleic, linoleic, and linolenic acids (Fig. 3) correlating with the expression of FAD2 (Fig. 2A), whose transcript level was undetectable at 32 DAA and at maturity (Fig. 2B). Sorensen *et al.* (42) observed the same trends up to 20 DAA in cultivars AC Emerson and Vimy. However, the level of ALA did not surpass 50% in these two cultivars at any stage of development, whereas AC McDuff consistently reaches levels of ALA between 50% and 60% regardless of the environmental conditions.

The genetic stability of genotypes is a key determinant of crop performance in the field. The phenotypic characterization of flax cv AC McDuff's developing bolls' FA content was assessed at two locations over two years. Environmental variations between years altered the FA profiles. From 8 DAA to maturity, the level of 18:1*cis*Δ9 was higher than that of 18:2*cis*Δ9,12 in 2003, compared with 2002, in which no difference could be observed between these two FA. The overall 18:3*cis*Δ9,12,15 content was slightly lower in 2003 compared with 2002, suggesting low desaturation efficiency of 18:2*cis*Δ9,12 into 18:3*cis*Δ9,12,15 in 2003. Delta-12 FA desaturase FAD2, which is known to be responsible for the desaturation of 18:1*cis*Δ9 into 18:2*cis*Δ9,12 (42), was shown to be transcriptionally regulated. Real-time PCR data revealed increased FAD2 overall transcript accumulation from 8 to 16 DAA, but 18:2*cis*Δ9,12 levels did not increase. However, 18:3*cis*Δ9,12,15 levels increased steadily, indicating that 18:2*cis*Δ9,12 is transient under normal weather conditions (2002), and FAD3, encoded also by two genes (15), converts 18:2*cis*Δ9,12 into 18:3*cis*Δ9,12,15 as quickly as FAD2 converts 18:1*cis*Δ9 into 18:2*cis*Δ9,12. This observation was further confirmed by FA profiles obtained under the warmer and dryer weather conditions of 2003. It seems that the high temperatures recorded in summer 2003 might have affected FAD2 activity. Consequently, a reduced oleic desaturation ratio (ODR) may have led to low substrate (18:2*cis*Δ9,12) availability for Δ-15 FA desaturase 3 (FAD3) enzymes involved in the desaturation of 18:2*cis*Δ9,12 to 18:3*cis*Δ9,12,15 (15). Green (25) reported that high temperatures considerably decreased the ODR and the relative proportions of linoleic and linolenic acids in controlled environments. Further studies will be needed to elucidate the role of temperature and environmental conditions on transcription, translation and post-translation control of FAD enzyme activity.

This study is the first, under field conditions, to examine flax FA profiles at different developmental stages and their correlation, within a single environment, with FA desaturase expression. The study suggested that FAD2, because of its key role in converting 18:1*cis*Δ9 into 18:2*cis*Δ9,12, is the most sensitive of the FA biosynthetic genes examined to environmental variations in the alteration of FA profiles under the field conditions investigated. SAD and FAD2 enzymes are also actively transcribed and translated at anthesis, suggesting that the desaturases may be involved in flower development, possibly by providing precursors of plant hormones such as jasmonic acid and oxylipins involved in the regulation of anthesis (44-46), or epicuticular wax biosynthesis which is required for inflorescence development and pollen fertility (47-50). Manipulation of these genes to produce designer flax with optimized FA content should take into consideration the roles played by these enzymes in other tissues (such as ovaries) as they may affect other essential biosynthetic pathways. Overall, this study provided evidence that SAD and FAD2 enzymes are highly expressed in ovaries and that 12–16 DAA constitutes a critical expression time for FA desaturase FAD2 in flax. This gene expression pattern was supported by phenotypic FA profiles that showed, independent of growing season, an increased accumulation of 18:3*cis*Δ9,12,15 by 12 DAA as a result of high activity of FA desaturases.

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Docosahexaenoic Acid Levels in the Lipids of Spotted Mackerel *Scomber australasicus*

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ABSTRACT: The lipid and FA compositions of various organs and of the stomach contents of *Scomber australasicus* were analyzed. DHA was characteristically the major FA of all the major lipid classes of all organs except for liver TAG. The mean DHA contents of the various organs accounted for more than 17% of the total FA (TFA), whereas those in the stomach contents, originating from the prey, fluctuated and were generally low. In particular, the DHA levels in the TAG from all organs of *S. australasicus* accounted for up to 17% of TFA, even though it is a neutral depot lipid. *S. australasicus* contained markedly high levels of DHA, even though it is a small-sized Scombridae species, and its high levels of DHA were close to those in large-sized highly migratory tuna species. Furthermore, DHA levels in its muscle TAG were consistently high, compared with those in the visceral TAG, which might be directly influenced by the prey lipids. These phenomena suggest that long-distance migration has a close relationship with high accumulation of DHA in fish tissues, since *S. australasicus* is reported to migrate in offshore water, similar to highly migratory tuna species. Additionally, the physiological selective accumulation of DHA in the muscle during migration is caused by *in vivo* metabolism of FA in the vascular system, suggesting that DHA is poorly used as a source of migration energy, though it is provided abundantly through the prey lipids.

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Occurrences of markedly high DHA contents in tissue lipids (more than 20% of crude total lipids [TL]) of large-sized tuna species belonging to the Scombridae family, such as *Thunnus thynnus* (1), *T. alalunga* (2,3), *T. albacares* (4), *Euthynnus pelamis* (1,5,6), *E. affinis* (7), *Auxis rochei*, and *A. thazard* (1,8), have been reported. Such fish actively and widely migrate in the offshore zones and exhibit different movement behavior from demersal, pelagic, coastal migratory, or reef fish. It has been hypothesized that these high levels are the result of accumulation of DHA during long-distance migration (7) because DHA is the most important essential FA in marine fishes (9,10) and is less effective than other FA as an energy source (11–13). On the other hand, DHA levels in the tissue lipids of nonmigratory fish, such as *Solea solea* (14), *Pagrus major* (15), *Lateolabrax japonicus* (15), *Seriola dumerili* (15), *Paralichthys olivaceus* (15), and *Caranx delicatissimus* (15), were reported to be relatively low (less than 20% of TL).

It is well known that DHA levels in the tissue phospholipids of all fish species are generally high, in comparison with the relatively low levels of n-3 PUFA in the neutral depot lipid TAG. In many cases, the FA composition of neutral lipids varies with environmental conditions, such as the stage of fish maturation, seawater temperature, and the lipid content of food sources (16,17). Moreover, in marine fishes, the DHA content in neutral lipids is generally less than 20% of the total FA (TFA), whereas saturated FA (SFA) and monounsaturated FA (MUFA), such as 16:0, 18:0, and 18:1n-9, have been found as major components.

On the other hand, tuna species characteristically contain high levels of DHA in the neutral lipids of orbital fats and head oils (see, for *T. obesus* and *E. pelamis*, (18); for *Thunnus* spp. and *E. pelamis*, (19)). There are no reports of high DHA content in neutral lipids except for the lipids of these tuna species, except for the report on muscle neutral lipids by Saito *et al.* (20). Recently, Saito *et al.* reported on the lipid of *Thunnus tonggol,* demonstrating that neutral lipids in muscle contained high levels of DHA compared with other organs (20). They suggested that comparatively high levels of DHA in muscle neutral lipids of the fish were caused by bioaccumulation of DHA during migration. Moreover, they also suggested that highly migratory fishes contain high levels of DHA in their muscle neutral lipids. In the case of tuna species, there are some reports on high levels of DHA in muscle TL compared with those in visceral TL, but there is no information on which lipid class of muscle TL contains most of the DHA, except for the report by Saito *et al*. We presumed that high levels of muscle DHA compared with visceral DHA may be due to high levels of DHA in muscle neutral lipids, as suggested by Saito *et al*. (20). It has been assumed that one of the reasons for the bioaccumulation of DHA observed in tuna species is that n-3 PUFA, including DHA, is poorly metabolized as an energy source compared with SFA or MUFA (21), and that DHA is left unused in the organs during the active metabolism associated with migration.

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Abbreviations: MUFA, monounsaturated FA; SE, steryl esters; SFA, saturated FA; ST, sterols; TFA, total FA; TL, total lipids; WE, wax esters.

From those previous findings, it seems likely that highly migratory fishes, even if they are not tuna species, contain high levels of DHA in their muscle neutral lipids.

Scomber australasicus, belonging to family Scombridae, is one of the most common fishes in Japan (together with *Scomber japonicus*), and has huge biomass in temperate to subtropical waters of the East China Sea. Although the body shape of the fish is similar to that of *S. japonicus*, the ecology of the two fish species differs. *S. australasicus* migrates widely throughout the tropical and temperate waters of the East China Sea (22–24), whereas the migration of *S. japonicus* is limited to coastal waters (22,24–26).

High DHA levels in the muscles of *S. australasicus* might be expected if the high level of DHA was caused by the active migration, even though the mean size of this species is not as large (24).

To determine the DHA levels in the various lipid classes of this species, and to estimate the relationship between its lipid physiology and ecology, the lipid classes and FA composition of its muscles, other internal organs, and stomach contents were analyzed in the present study.

MATERIALS AND METHODS

Materials. Sampling locations and body dimensions of *Scomber australasicus* examined in the present study are listed in Table 1. The specimens were caught in the east China Sea (34°09′N, 127°39′E and 34°55′N, 132°10′E) by using set-net in October 2002 (sample no. 1) and in December 2004 (sample no. 2). Both the samples were brought to our laboratory under ice-storage condition on the following day of catch. The biological data of the sample 1 specimens (fork length: 277 ± 7 mm, body weight: 272 ± 27 g, $n = 5$) and sample 2 specimens (fork length: 335 ± 9 mm, body weight: 567 ± 54 g, $n = 6$) were measured. The fish were immediately frozen and kept at −80°C for 2 wk to 1 mon prior to lipid extraction.

Lipid extraction and the analysis of lipid classes. Frozen fish were partially thawed, and their dorsal ordinary muscles (white muscle), liver, and other viscera (without stomach contents and gill) were cut out using a scalpel after wiping. The stomach contents were then removed and used as samples. Each sample was homogenized in a mixture of chloroform and methanol (2:1, vol/vol), and a portion of each homogenized sample was extracted according to the Folch *et al.* procedure (27). The crude TL (50–100 mg) were separated into classes on silicic acid columns (Kieselgel 60, 70–230 mesh, Merck and Co. Ltd., Darmstadt, Germany), and a quantitative analysis of the lipid constituents was performed using gravimetric analysis of fractions collected from column chromatography. The first eluate (dichloromethane/*n*-hexane, 2:3, vol/vol) was collected as the steryl ester (SE), which contained a small amount of wax ester (WE). This was followed with: dichloromethane eluting the TAG; dichloromethane/ether (35:1, vol/vol) eluting the sterols (ST); dichloromethane/ether (9:1, vol/vol) eluting the DG; dichloromethane/methanol (9:1, vol/vol) eluting the FFA; dichloromethane/methanol (1:1, vol/vol), eluting the PE; dichloromethane/methanol (1:5, vol/vol), eluting the other minor phospholipids; and dichloromethane/methanol (1:20, vol/vol) to elute the PC (28). Individual lipids from each lipid class were identified with authentic samples by comparison of *Rf* values using TLC (Merck & Co. Ltd., Kieselgel 60, thickness of 0.25 mm for analysis) (29). All sample lipids were dried under nitrogen at room temperature and stored at −80°C.

Preparation of methyl esters and GLC of the esters. The TAG, PE, and PC fractions were directly trans-esterified with boiling methanol containing 1% of concentrated hydrochloric acid under reflux for 1.5 h as previously reported to produce the FAME (30). These methyl esters were purified using silica gel column chromatography by elution with dichloromethane.

Analyses of FAME were performed on a gas chromatograph (GC–17A, Shimadzu Seisakusho Co., LTD, Kyoto, Japan) equipped with a capillary column (Omegawax–250, 30m × 0.25 mm i.d., 0.25 μm film thickness; split ratio, 20:1; Supelco Japan Co., Ltd, Tokyo, Japan). The temperatures of the injector, column, and detector were maintained at 250°C, 205°C, and 250°C, respectively. Helium was used as carrier gas at a constant inlet rate of 0.8 mL/min. Linear velocity of the carrier gas was 26.2 cm/s.

Quantitation of individual components was performed by means of Shimadzu Model C-R7A (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan) electronic integrators. The weight percentages of individual components in the lipids were expressed as relative percentage of peak area to total peak area.

Analysis of FAME by GC–MS. Analysis of the FAME was performed on GC–MS QP 2010 (Shimadzu Seisakusho Co., LTD, Kyoto, Japan) GC–MS equipped with the same capillary. The temperatures of the injector and the column were held at 240°C and 210°C, respectively. The split ratio was 1:76, and the ionization voltage was 70 eV. Helium was used as the carrier gas at a constant inlet rate of 0.7 mL/min.

FAME were identified using, (i) marine lipid methyl esters as standards (Supelco 37 Component FAME Mix, Supelco Japan Ltd., Tokyo, Japan) and (ii) by comparison of mass spectral data obtained by GC–MS.

Statistical analyses. Significant differences in the data of the total SFA, total MUFA, total n-6 PUFA, and total n-3 PUFA of the classes (TAG, PE, and PC) of samples 1 and 2 were determined using one-way ANOVA at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Lipid content with biological data. The biological data of the specimens examined and their TL contents are given in Tables 1 and 2. The body sizes of each specimen from sample 1 were smaller than those of sample 2. In each organ, the TL contents of sample 1 (muscle, 1.3%; pyloric cecum, 8.2%; other viscera, 8.1%) were significantly lower than those of sample 2 (muscle, 5.3%; pyloric cecum, 13.8%; other viscera, 28.3%), except for that in liver lipids (sample 1, 9.4 ± 1.5%; sample 2, 11.8 ± 3.1%).

In both samples, the TL contents of muscle (1.3–5.3%) were much lower than those of other tissues (liver, 9.4–11.8%;

TABLE 1
Locality of Capture and Biological Data of *S. australasicus* Examined[a]

Scientific name	Sampling no.	Date	Locality	Replicate animals (*n*)	Fork length (mm)	Body weight (g)
Scomber australasicus	1	October 7, 2002	34°09'N 127°39'E	5	276.6 ± 7.7	271.9 ± 26.7
	2	December 13, 2004	34°55'N 132°10'E	6	334.5 ± 9.3	567.4 ± 53.8

[a]Results are expressed as mean ± SD (n = 5 or 6).

pyloric cecum, 8.2–13.8%; other viscera, 8.1–28.3%). In comparison with other migratory fishes belonging to family Scombridae, the content of muscle TL of *S. australasicus* was higher than those of tuna species reported in the literature: *Thunnus obesus,* 0.8 ± 0.3% (1); *T. albacares,* 0.6 ± 0.0% (1) and 0.5 ± 0.3% (4); and *T. thynnus,* 1.0 ± 0.5% (1) and 1.4 ± 0.6% (31). However, the content of *S. australasicus* muscle TL was lower than those of *S. japonicus* (32) caught during autumn to winter (2.8–18.9%). These findings may suggest that *S. australasicus* and *S. japonicus* can use their muscles for lipid storage, as other small-sized pelagic fishes do (33,34).

Lipid classes of the respective organs of S. australasicus. In all the organs, the lipids mainly contained TAG, sterols (ST), and FFA in the neutral depot lipids, and PE and PC in the polar tissue lipids, with small levels of SE, WE, and DAG (Table 2).

The total lipids of all the specimens consistently contained high levels of the glycerol derivatives, such as TAG, PE, and PC. The total amounts of the three major classes (TAG, PE, and PC) were more than 50% (71.9–93.5% for the muscles, 55.4–92.0% for the viscera) of TL in all of the organs. These high levels of glycerol derivatives might be a typical characteristic of marine fish species, similar to those in other fish species. The TAG levels of each organ from sample 1 (36.5–55.2%) were statistically lower than those contained in each organ of sample 2 (69.0–90.4%). This suggests that the TL contents in the tissues vary due to TAG composition, whereas polar lipid contents, such as PE or PC, do not vary.

Moderate levels of FFA (2.6–24.5%) were found in the internal organs; in particular, the digestive organs (pyloric cecum and liver). This may be a result of the degradation of glycerol derivatives, such as TAG and phospholipids, by enzymatic metabolism; the high FFA levels were mainly found in digestive viscera that may have active enzymes for digestion. Similar tendencies were reported for *T. tonggol* internal organs by Saito *et al*. (20). Sterols were found at moderate levels (2.4–8.8%) in all samples.

FA composition in TL and TAG. The FA compositions of the three major components (TAG, PE, and PC) with TL in each class, separated from the various organs of *S. australasicus*, are shown in Tables 3–6. At least 50 FA components were detected and identified, of which around 40 compounds occurred at a level of 0.1% or more of TFA. In the FA of TL, only five components were found as major FA of all the organs: two SFA, 16:0 (20.3–24.4%) and 18:0 (5.3–11.1%); one MUFA, 18:1 n-9 (10.2–29.4%); and two n-3 PUFA, 20:5 n-3 (EPA, 3.2–8.2%) and DHA (9.1–27.5%), as shown in Table 3. These main FA were detected at levels of about 5% or more in almost all of the organs. Noticeable amounts (>mean 2%) of several other FA for both of two replicate samples 1 and 2, 14:0 (0.9–4.7%), 16:1n-7 (1.8–5.2%), 18:1n-7 (2.1–4.7%), and 20:1n-9 (1.0–3.7%), were found. There was only one exception; the DHA levels in the liver of the sample 2 were extremely low (9.1 ± 2.5%). In the stomach contents, FA compositions fluctuated between the samples, and the DHA levels of sample 1

TABLE 2
Lipid Content and Lipid Classes of *S. australasicus*[a]

Sample no. (year collected)	Lipid content[b]	SE[c,d]	TAG[b,c]	ST[c]	DG[c]	FFA[c]	PE[c]	OPL[c]	PC[c]
1 (2002)[e]									
White muscle	1.3 ± 0.5[f]	2.5 ± 1.2	36.5 ± 14.2[d]	8.3 ± 2.2	3.1 ± 0.2	7.2 ± 2.3	16.0 ± 3.2	7.1 ± 4.5	19.4 ± 5.9
Liver	9.4 ± 1.5[c,d]	3.4 ± 1.0	55.2 ± 8.9[c]	8.6 ± 0.9	5.4 ± 2.3	11.2 ± 3.1	7.1 ± 2.9	3.4 ± 1.6	5.7 ± 2.3
Pyloric cecum	8.2 ± 2.5[d,e]	2.2 ± 1.3	45.8 ± 13.8[c,d]	8.8 ± 3.0	7.4 ± 1.1	24.5 ± 5.6	6.5 ± 2.6	1.7 ± 0.4	3.1 ± 2.1
Other viscera	8.1 ± 3.0[d,e]	2.3 ± 0.8	48.1 ± 9.5[c,d]	7.5 ± 1.5	7.1 ± 0.7	23.1 ± 4.7	6.5 ± 2.5	2.2 ± 0.8	3.2 ± 0.9
Stomach contents	1.1 ± 0.4								
2 (2004)[f]									
White muscle	5.3 ± 2.2[e]	0.4 ± 0.2	88.0 ± 3.9[a]	2.4 ± 0.7	0.7 ± 0.3	1.2 ± 0.6	2.3 ± 1.0	1.7 ± 1.0	3.2 ± 0.9
Liver	11.8 ± 3.1[b,c]	1.7 ± 0.4	69.0 ± 7.9[b]	4.4 ± 1.1	3.6 ± 0.6	10.9 ± 3.4	3.8 ± 1.2	2.8 ± 1.3	3.8 ± 1.1
Pyloric cecum	13.8 ± 2.3[b]	0.6 ± 0.2	79.7 ± 3.5[a,b]	4.3 ± 0.4	2.2 ± 0.2	9.0 ± 2.1	1.8 ± 0.6	1.1 ± 0.9	1.2 ± 0.8
Other viscera	28.3 ± 4.2[a]	0.3 ± 0.1	90.4 ± 1.8[a]	3.6 ± 2.0	1.2 ± 0.3	2.6 ± 0.9	1.0 ± 0.3	0.3 ± 0.1	0.7 ± 0.4
Stomach contents	1.3 ± 0.2								

[a]Results are expressed as weight percent of wet tissues.
[b]Different superscripts indicate statistical differences ($P < 0.05$).
[c]Results are expressed as weight percent of total lipids. SE, steryl esters; TAG, triacylglycerols; ST, sterols; DG, diacylglycerols; FFA, free fatty acids. PE, phosphatidylethanolamine; PC, phosphatidylcholine; OPL, other phospholipids.
[d]The SE fraction of both samples contained small amounts of wax esters.
[e]Data are mean ± SD ($n = 5$).
[f]Data are mean ± SD ($n = 6$).

TABLE 3
FA Composition of TL in Organs of *S. australasicus*[a,b]

	White muscle		Liver		Pyloric cecum		Other viscera		Stomach contents	
Sample no.	1	2	1	2	1	2	1	2	1	2
Total saturated	34.6 ± 2.5	33.5 ± 0.9	30.3 ± 2.0	32.7 ± 2.8	39.3 ± 1.8	35.8 ± 1.1	39.2 ± 1.5	33.3 ± 1.2	38.9 ± 3.3	38.1 ± 3.2
14:0	1.7 ± 0.5	4.2 ± 0.1	1.4 ± 0.4	0.9 ± 0.1	3.5 ± 1.0	4.7 ± 0.1	3.8 ± 0.9	4.6 ± 0.2	2.7 ± 0.3	4.5 ± 0.6
15:0	0.8 ± 0.2	1.2 ± 0.1	0.9 ± 0.3	0.3 ± 0.1	1.3 ± 0.2	1.4 ± 0.1	1.4 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
16:0	22.7 ± 1.7	21.6 ± 0.9	20.6 ± 1.7	24.4 ± 2.6	23.6 ± 0.7	21.8 ± 0.8	23.5 ± 1.1	20.3 ± 1.0	21.9 ± 1.6	23.0 ± 2.4
17:0	1.2 ± 0.2	1.2 ± 0.1	1.6 ± 0.4	1.0 ± 0.1	1.8 ± 0.1	1.2 ± 0.1	1.8 ± 0.1	1.4 ± 0.2	2.1 ± 0.4	0.8 ± 0.1
18:0	8.2 ± 0.7	5.3 ± 0.4	5.8 ± 1.0	6.0 ± 0.6	9.0 ± 0.7	6.7 ± 0.4	8.7 ± 0.4	5.8 ± 0.2	11.1 ± 1.8	8.7 ± 1.4
Total monoenoic	15.4 ± 3.7	22.6 ± 1.6	27.6 ± 7.1	41.0 ± 4.4	17.0 ± 3.1	19.7 ± 1.3	18.7 ± 3.5	23.0 ± 1.4	18.8 ± 2.9	14.8 ± 1.9
16:1n-7	1.8 ± 0.4	4.9 ± 0.4	1.9 ± 0.5	3.2 ± 0.6	2.7 ± 1.0	4.6 ± 0.4	2.5 ± 0.4	5.2 ± 0.4	3.0 ± 0.6	3.6 ± 0.5
18:1n-9	10.2 ± 2.5	13.2 ± 0.9	17.4 ± 5.6	29.4 ± 3.8	10.3 ± 2.1	10.9 ± 0.7	11.7 ± 2.8	13.2 ± 0.8	12.0 ± 1.8	7.6 ± 1.1
18:1n-7	2.1 ± 0.2	2.7 ± 0.2	3.8 ± 0.4	4.7 ± 0.4	2.1 ± 0.0	2.6 ± 0.2	2.3 ± 0.1	2.9 ± 0.2	2.3 ± 0.3	2.5 ± 0.4
20:1n-9	1.0 ± 0.6	1.2 ± 0.2	3.7 ± 1.6	2.9 ± 0.9	1.4 ± 0.5	1.0 ± 0.2	1.6 ± 0.6	1.2 ± 0.2	1.0 ± 0.3	0.8 ± 0.3
22:1n-11	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n-9	0.3 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.6 ± 0.1	0.4 ± 0.2	0.3 ± 0.1
24:1n-9	0.4 ± 0.2	0.1 ± 0.2	0.6 ± 0.3	0.2 ± 0.2	0.6 ± 0.3	0.1 ± 0.2	0.8 ± 0.0	0.2 ± 0.2	0.8 ± 0.3	0.1 ± 0.2
Total polyenoic	41.2 ± 5.6	35.2 ± 1.9	31.0 ± 6.8	19.1 ± 4.6	33.3 ± 3.0	34.7 ± 1.1	31.4 ± 2.5	35.0 ± 0.8	32.9 ± 4.3	38.2 ± 4.8
n-6 series	6.7 ± 0.7	5.1 ± 0.5	7.1 ± 0.9	3.6 ± 0.6	7.1 ± 0.7	5.4 ± 0.4	6.9 ± 0.6	5.2 ± 0.5	10.8 ± 0.8	7.2 ± 0.5
18:2n-6	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	0.6 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.4 ± 0.1	1.5 ± 0.2
20:4n-6	3.2 ± 0.4	2.8 ± 0.5	3.0 ± 0.2	1.9 ± 0.3	3.4 ± 0.6	2.9 ± 0.3	3.1 ± 0.4	2.6 ± 0.4	5.2 ± 0.5	4.4 ± 0.4
22:4n-6	0.4 ± 0.1	0.3 ± 0.0	1.4 ± 0.3	0.7 ± 0.2	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	1.2 ± 0.2	0.5 ± 0.1
22:5n-6	1.9 ± 0.2	0.9 ± 0.1	1.8 ± 0.3	0.3 ± 0.1	1.9 ± 0.1	1.0 ± 0.1	2.0 ± 0.2	1.1 ± 0.2	3.0 ± 0.3	0.9 ± 0.1
n-3 series	34.5 ± 5.2	30.1 ± 2.0	23.9 ± 6.0	15.6 ± 4.0	26.2 ± 2.6	29.4 ± 1.2	24.5 ± 2.1	29.7 ± 1.1	22.2 ± 4.2	30.9 ± 4.6
18:4n-3	0.3 ± 0.1	1.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.1	1.3 ± 0.2	0.6 ± 0.1	1.9 ± 1.2	0.4 ± 0.2	1.2 ± 0.4
20:4n-3	0.2 ± 0.0	0.5 ± 0.0	0.5 ± 0.2	0.7 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
20:5n-3	5.1 ± 0.5	8.1 ± 0.4	3.2 ± 0.6	3.2 ± 0.6	5.4 ± 0.5	7.8 ± 0.5	5.1 ± 0.6	8.2 ± 0.5	4.8 ± 1.2	8.4 ± 1.2
22:5n-3	1.4 ± 0.1	1.7 ± 0.1	4.4 ± 1.5	2.4 ± 0.7	1.4 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.8 ± 0.1	1.3 ± 0.3	1.2 ± 0.1
22:6n-3	27.5 ± 4.9[a]	18.6 ± 2.0[b,c]	15.6 ± 3.8[c]	9.1 ± 2.5[d]	18.5 ± 2.1[b,c]	18.2 ± 1.2[b,c]	17.0 ± 1.4[b,c]	17.3 ± 0.9[b,c]	15.4 ± 2.9[c]	19.8 ± 3.4[b]
Total FA	91.6 ± 1.1	91.4 ± 0.4	89.5 ± 2.0	93.0 ± 2.6	90.3 ± 0.7	90.3 ± 1.4	90.1 ± 0.4	91.4 ± 0.8	91.4 ± 0.4	91.0 ± 1.2

[a]Results are expressed as weight percent of total FA. Data are mean ± SD for several samples (n = 5–6).
[b]Different superscript in the row of 22:6n-3 indicate statistical differences ($P < 0.05$).

TABLE 4
FA Composition of TAG in Organs of S. australasicus[a,b]

	White muscle		Liver		Pyloric cecum		Other viscera	
Sample no.	1	2	1	2	1	2	1	2
Total saturated	32.7 ± 2.2	33.8 ± 1.1	27.1 ± 0.9	33.6 ± 3.4	37.9 ± 1.3	35.9 ± 1.1	38.4 ± 1.9	34.8 ± 1.0
14:0	3.4 ± 0.6	4.6 ± 0.2	1.6 ± 0.5	1.1 ± 0.1	4.2 ± 0.9	5.1 ± 0.2	4.3 ± 0.9	4.7 ± 0.2
15:0	1.2 ± 0.3	1.3 ± 0.1	0.9 ± 0.3	0.4 ± 0.1	1.5 ± 0.2	1.4 ± 0.1	1.5 ± 0.3	1.3 ± 0.1
16:0	20.4 ± 1.7	21.7 ± 1.0	17.9 ± 1.3	25.2 ± 3.2	22.5 ± 0.9	21.7 ± 0.9	23.0 ± 1.8	21.3 ± 1.0
17:0	1.4 ± 0.2	1.3 ± 0.1	2.0 ± 0.4	1.1 ± 0.1	1.7 ± 0.2	1.3 ± 0.1	1.8 ± 0.1	1.3 ± 0.1
18:0	6.4 ± 0.6	4.9 ± 0.3	4.7 ± 0.9	5.8 ± 0.6	8.0 ± 0.1	6.4 ± 0.4	7.8 ± 0.3	6.1 ± 0.3
Total monoenoic	21.0 ± 5.1[c,d]	23.6 ± 1.4[b,c]	30.9 ± 8.4[b]	46.6 ± 3.3[a]	18.8 ± 3.0[d]	21.0 ± 0.9[c,d]	19.9 ± 4.4[c,d]	22.6 ± 0.9[c,d]
16:1n-7	3.5 ± 1.6	5.1 ± 0.5	2.1 ± 0.4	3.3 ± 0.8	2.9 ± 0.8	4.7 ± 0.4	2.7 ± 0.3	4.8 ± 0.4
18:1n-9	13.4 ± 3.7	13.8 ± 0.8	19.6 ± 6.8	33.5 ± 3.2	11.4 ± 2.5	11.8 ± 0.8	12.3 ± 3.5	13.1 ± 0.8
18:1n-7	2.3 ± 0.4	2.8 ± 0.1	4.3 ± 0.3	5.4 ± 0.3	2.2 ± 0.1	2.7 ± 0.2	2.3 ± 0.2	2.9 ± 0.2
20:1n-9	1.4 ± 0.6	1.3 ± 0.2	4.1 ± 1.7	3.4 ± 1.1	1.7 ± 0.6	1.1 ± 0.2	1.9 ± 0.8	1.3 ± 0.2
22:1n-11	0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.0	0.4 ± 0.5	0.1 ± 0.1	0.2 ± 0.3	0.1 ± 0.1	0.2 ± 0.3
22:1n-9	0.4 ± 0.2	0.6 ± 0.2	0.8 ± 0.1	0.7 ± 0.4	0.6 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
24:1n-9	0.7 ± 0.1	0.2 ± 0.2	0.6 ± 0.3	0.3 ± 0.3	0.8 ± 0.0	0.4 ± 0.2	0.7 ± 0.0	0.2 ± 0.2
Total polyenoic	30.8 ± 2.8	33.7 ± 1.5	27.2 ± 8.1	13.9 ± 3.9	28.9 ± 2.0	33.1 ± 1.5	28.3 ± 3.2	33.2 ± 1.0
n-6 series	5.4 ± 1.1	4.8 ± 0.5	6.0 ± 1.4	2.7 ± 0.5	5.7 ± 0.3	4.9 ± 0.4	5.5 ± 0.7	4.9 ± 0.5
18:2n-6	1.2 ± 0.3	1.2 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.1
20:4n-6	2.1 ± 0.4	2.6 ± 0.5	2.0 ± 0.4	1.3 ± 0.2	2.4 ± 0.2	2.4 ± 0.3	2.1 ± 0.3	2.4 ± 0.3
22:4n-6	0.4 ± 0.1	0.3 ± 0.0	1.5 ± 0.4	0.7 ± 0.2	0.5 ± 0.1	0.2 ± 0.2	0.5 ± 0.1	0.3 ± 0.1
22:5n-6	1.7 ± 0.4	0.8 ± 0.1	1.6 ± 0.5	0.1 ± 0.1	1.7 ± 0.2	1.1 ± 0.2	1.7 ± 0.3	1.1 ± 0.2
n-3 series	25.4 ± 2.1	28.8 ± 1.8	21.2 ± 6.8	11.1 ± 3.6	23.1 ± 1.7	28.2 ± 1.8	22.8 ± 2.5	28.2 ± 1.4
18:4n-3	0.8 ± 0.2	1.5 ± 0.2	0.3 ± 0.1	0.2 ± 0.0	0.7 ± 0.1	1.4 ± 0.2	0.7 ± 0.1	1.4 ± 0.2
20:4n-3	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
20:5n-3	5.0 ± 0.6	8.2 ± 0.5	2.7 ± 0.8	2.4 ± 0.6	4.9 ± 0.5	7.5 ± 0.5	4.8 ± 0.6	7.7 ± 0.4
22:5n-3	1.7 ± 0.1	1.7 ± 0.1	4.7 ± 1.7	2.5 ± 0.9	1.4 ± 0.1	1.6 ± 0.1	1.5 ± 0.2	1.7 ± 0.1
22:6n-3	17.4 ± 1.6[a]	17.0 ± 1.9[a]	12.9 ± 4.3[b]	5.4 ± 2.0[c]	15.8 ± 1.1[a]	17.3 ± 1.6[a]	15.5 ± 1.8[a,b]	17.0 ± 1.2[a]
Total FA	85.2 ± 5.0	91.2 ± 1.0	85.8 ± 1.6	94.3 ± 0.9	86.3 ± 2.1	90.4 ± 1.0	87.3 ± 1.3	90.8 ± 0.9

[a]Results are expressed as weight percent of total FA. Data are mean ± SD for several samples (n = 5–6).
[b]Different superscripts in the same row for total monoenoic and 22:6n-3 indicate statistical differences ($P < 0.05$).

TABLE 5
FA Composition of PE in Organs of *S. australasicus*[a]

	White muscle		Liver		Pyloric cecum		Other viscera	
Sample no.	1	2	1	2	1	2	1	2
Total saturated	25.2 ± 2.6	25.3 ± 2.0	31.7 ± 3.4	29.7 ± 2.1	35.4 ± 2.9	29.0 ± 2.2	35.9 ± 3.5	30.7 ± 2.3
14:0	0.3 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	0.2 ± 0.0	1.8 ± 0.7	1.2 ± 0.4	2.2 ± 0.8	1.5 ± 0.2
15:0	0.2 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	0.2 ± 0.0	0.9 ± 0.2	0.7 ± 0.1	0.9 ± 0.2	0.6 ± 0.1
16:0	7.1 ± 0.7	7.6 ± 0.7	16.2 ± 1.9	13.6 ± 1.1	17.5 ± 1.8	14.3 ± 1.2	17.1 ± 2.5	14.1 ± 1.6
17:0	1.2 ± 0.2	0.5 ± 0.3	1.5 ± 0.3	0.3 ± 0.1	1.8 ± 0.4	0.8 ± 0.4	1.9 ± 0.2	0.9 ± 0.1
18:0	16.5 ± 1.8	16.7 ± 1.2	12.6 ± 1.4	15.4 ± 1.2	13.3 ± 2.5	12.0 ± 0.4	13.8 ± 1.8	13.6 ± 0.8
Total monoenoic	10.3 ± 1.1	10.2 ± 1.1	14.0 ± 3.3	12.9 ± 1.6	10.9 ± 1.7	9.1 ± 1.0	14.7 ± 2.7	13.4 ± 1.3
16:1n-7	1.2 ± 0.2	1.3 ± 0.3	1.0 ± 0.2	0.6 ± 0.2	1.7 ± 0.3	1.7 ± 0.5	1.8 ± 0.4	2.0 ± 0.4
18:1n-9	5.0 ± 0.5	5.0 ± 0.6	8.2 ± 1.9	7.8 ± 1.0	6.3 ± 1.0	4.6 ± 0.5	8.9 ± 2.0	7.4 ± 0.8
18:1n-7	3.4 ± 0.5	3.4 ± 0.2	2.4 ± 0.5	2.6 ± 0.3	2.1 ± 0.3	2.4 ± 0.1	2.5 ± 0.3	3.2 ± 0.2
20:1n-9	0.6 ± 0.3	0.4 ± 0.1	2.2 ± 0.9	1.9 ± 0.5	0.8 ± 0.3	0.4 ± 0.0	1.1 ± 0.4	0.7 ± 0.1
22:1n-11	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n-9	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
24:1n-9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	0.5 ± 0.1	0.0 ± 0.0
Total polyenoic	47.9 ± 2.2	57.2 ± 2.9	39.8 ± 5.5	52.1 ± 3.2	33.9 ± 2.8	53.0 ± 2.7	29.6 ± 3.1	47.5 ± 3.7
n-6 series	8.4 ± 2.3	8.3 ± 0.8	12.9 ± 1.2	11.6 ± 0.7	8.3 ± 1.1	11.0 ± 0.6	9.2 ± 1.2	12.3 ± 1.4
18:2n-6	1.5 ± 0.6	1.4 ± 0.1	1.0 ± 0.2	0.6 ± 0.1	1.2 ± 0.2	0.9 ± 0.1	1.3 ± 0.1	0.9 ± 0.1
20:4n-6	5.0 ± 2.6	4.8 ± 0.6	8.8 ± 0.9	8.7 ± 0.5	4.9 ± 0.8	8.5 ± 0.6	4.9 ± 1.0	8.5 ± 1.1
22:4n-6	0.4 ± 0.3	0.3 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.2 ± 0.2	0.3 ± 0.1	0.8 ± 0.1	0.9 ± 0.2
22:5n-6	1.4 ± 0.1	1.8 ± 0.1	2.2 ± 0.3	1.5 ± 0.1	2.1 ± 0.3	1.3 ± 0.1	2.3 ± 0.2	1.9 ± 0.3
n-3 series	39.5 ± 3.0	49.0 ± 3.3	26.9 ± 4.9	40.5 ± 3.1	25.5 ± 2.1	42.0 ± 2.4	20.3 ± 2.9	35.2 ± 2.9
18:4n-3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.0	0.2 ± 0.2	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
20:4n-3	0.2 ± 0.1	0.3 ± 0.0	0.7 ± 0.1	1.1 ± 0.2	0.6 ± 1.0	0.3 ± 0.2	0.3 ± 0.0	0.5 ± 0.1
20:5n-3	2.9 ± 0.8	4.0 ± 0.4	2.4 ± 0.5	4.0 ± 0.4	2.9 ± 0.2	5.6 ± 0.4	2.9 ± 0.6	5.4 ± 0.3
22:5n-3	1.4 ± 0.7	1.5 ± 0.2	2.7 ± 0.5	2.6 ± 0.4	1.0 ± 0.1	1.8 ± 0.3	1.1 ± 0.2	2.2 ± 0.4
22:6n-3	34.9 ± 3.9	43.0 ± 3.6	20.9 ± 4.2	32.6 ± 2.9	21.0 ± 1.7	33.9 ± 1.9	15.7 ± 2.4	26.9 ± 2.5
Total FA	83.4 ± 1.6	92.7 ± 1.3	85.5 ± 1.1	94.7 ± 0.8	80.3 ± 5.8	91.0 ± 1.4	80.6 ± 2.5	91.6 ± 1.5

[a]Results are expressed as weight percent of total FA. Data are mean ± SD for several samples (n = 5–6)

were lower than those of the organs, whereas the levels of sample 2 were almost the same as those of the organs.

The remarkably high DHA levels (18.6–27.5%) observed in muscle TL of *S. australasicus* were comparable to those of highly migratory tuna species, such as *Thunnus thynnus* (32.1%) (1), *T. alalunga* (24.3–25.9%) (2,3), *T. albacares* (27.3–36.0%) (4), *E. pelamis* (33.3%) (1,5,6), *E. affinis* (21.3%) (7), *Auxis rochei* (19.8%), and *A. thazard* (29.1%) (1,8). Moreover, the DHA levels were much higher than those of *S. japonicus* (10.5–18.1%) (32) or *Scomberomorus commerson* (8.4–8.6%) (35), although their body shape is very similar to that of *S. australasicus* (22–26).

It is known that marine fishes are unable to synthesize DHA (36–43). The characteristically high levels of DHA observed in highly migratory tuna species have been explained by their highest trophic position in the marine grazing food chain, as well as their high migration with active consumption of MUFA and SFA and accumulation of n-3 PUFA.

In the FA of TAG, only five components were found as major FA of all the organs, similar to those observed in the FA of TL: two SFA, 16:0 (17.9–25.2%) and 18:0 (4.7–8.0%); one MUFA, 18:1n-9 (11.4–33.5%); and two n-3 PUFA, EPA (2.4–8.2%) and DHA (5.4–17.4%), as shown in Table 4. Noticeable amounts of several other FA were found in both of two replicate samples 1 and 2: 14:0 (1.1–5.0%), 16:1n-7 (2.1–5.1%), 18:1n-7 (2.2–5.4%), and 20:1n-9 (1.1–4.1%). There was only one exception; the DHA levels in the liver of sample 2 were extremely low (5.4 ± 2.0%), whereas the MUFA levels of the sample were higher than those in the other organs' TAG.

The DHA levels in the TAG of the muscles were significantly high (17.0–17.4%) compared with the liver TAG (5.4–12.9%), and did not fluctuate between samples, whereas those in liver TAG fluctuated between samples. Although there were no significant differences in DHA levels among muscle, pyloric cecum, and other viscera, mean DHA levels in muscle TAG were highest, and the levels in pyloric cecum and other viscera fluctuated between the samples. The consistently high DHA levels of muscle TAG may be characteristic of highly migratory fishes belonging to Scombridae, and it may suggest that in these species DHA was selectively accumulated through the internal organs. The MUFA levels in the liver from both samples were higher than those of prey lipids, as well as liver of fatty sample 2 contained higher levels of MUFA than those contained in the liver of lean sample 1. Such phenomena observed in the liver TAG may suggest that the liver plays a role

TABLE 6
FA Composition of PC in Organs of *S. australasicus*[a]

	White muscle		Liver		Pyloric cecum		Other viscera	
Sample no.	1	2	1	2	1	2	1	2
Total saturated	32.8 ± 1.3	33.1 ± 1.1	36.2 ± 2.8	40.4 ± 1.9	34.9 ± 3.4	35.1 ± 2.3	35.9 ± 2.1	36.8 ± 3.4
14:0	0.4 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	1.1 ± 0.3	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.2
15:0	0.7 ± 0.1	0.7 ± 0.1	1.0 ± 0.3	0.4 ± 0.1	1.2 ± 0.2	1.5 ± 0.1	1.2 ± 0.2	1.2 ± 0.1
16:0	28.2 ± 1.1	29.2 ± 1.0	29.8 ± 2.4	34.6 ± 1.7	25.2 ± 4.0	25.6 ± 2.0	25.6 ± 1.6	27.9 ± 2.2
17:0	0.6 ± 0.1	0.3 ± 0.0	1.0 ± 0.2	0.4 ± 0.1	1.7 ± 0.3	1.3 ± 0.2	1.7 ± 0.1	1.4 ± 0.1
18:0	2.8 ± 0.5	2.1 ± 0.2	3.7 ± 0.4	4.5 ± 0.5	5.7 ± 0.6	4.7 ± 0.8	6.1 ± 0.5	4.9 ± 0.8
Total monoenoic	10.1 ± 0.7	11.7 ± 1.0	9.9 ± 1.5	7.4 ± 0.4	12.0 ± 1.1	12.1 ± 1.3	14.9 ± 0.8	14.6 ± 1.7
16:1n-7	0.7 ± 0.1	1.2 ± 0.1	1.6 ± 0.5	0.8 ± 0.3	1.7 ± 0.6	2.1 ± 0.5	1.4 ± 0.1	2.1 ± 0.6
18:1n-9	8.0 ± 0.6	9.0 ± 0.8	6.4 ± 1.0	5.1 ± 0.3	7.7 ± 0.6	7.1 ± 0.7	10.4 ± 0.7	9.4 ± 1.1
18:1n-7	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	2.1 ± 0.2	2.5 ± 0.2	2.5 ± 0.1	2.7 ± 0.2
20:1n-9	0.2 ± 0.1	0.2 ± 0.0	0.8 ± 0.5	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.1
22:1n-11	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-9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
24:1n-9	0.0 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.3 ± 0.1	0.1 ± 0.2
Total polyenoic	51.7 ± 1.5	52.0 ± 1.7	46.1 ± 3.5	49.6 ± 1.8	41.8 ± 4.3	44.5 ± 3.0	37.7 ± 3.3	42.0 ± 5.2
n-6 series	8.1 ± 0.8	7.8 ± 0.6	10.2 ± 0.9	6.4 ± 0.2	10.9 ± 1.5	8.1 ± 0.5	11.3 ± 1.5	9.3 ± 0.9
18:2n-6	0.7 ± 0.0	0.7 ± 0.0	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
20:4n-6	4.5 ± 0.5	5.1 ± 0.6	5.9 ± 0.8	4.0 ± 0.2	7.1 ± 0.8	6.4 ± 0.5	7.1 ± 1.0	6.7 ± 0.7
22:4n-6	0.4 ± 0.1	0.3 ± 0.0	1.0 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
22:5n-6	2.5 ± 0.3	1.7 ± 0.1	2.3 ± 0.2	1.0 ± 0.1	2.2 ± 0.6	0.4 ± 0.4	2.4 ± 0.4	1.0 ± 0.2
n-3 series	43.6 ± 1.7	44.1 ± 2.0	35.8 ± 3.8	43.2 ± 1.7	30.9 ± 2.8	36.4 ± 2.6	26.4 ± 1.9	32.7 ± 4.6
18:4n-3	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.5 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.4 ± 0.1
20:4n-3	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.1
20:5n-3	7.2 ± 0.5	12.0 ± 1.0	6.9 ± 0.7	9.5 ± 0.5	8.4 ± 1.0	12.4 ± 1.0	7.1 ± 0.4	10.9 ± 1.9
22:5n-3	1.3 ± 0.1	1.4 ± 0.1	2.6 ± 0.4	1.7 ± 0.2	1.3 ± 0.4	1.2 ± 0.6	1.4 ± 0.2	1.9 ± 0.2
22:6n-3	34.9 ± 1.9	30.2 ± 2.8	25.8 ± 3.1	31.0 ± 1.8	20.8 ± 3.0	22.1 ± 1.3	17.4 ± 1.7	19.2 ± 2.6
Total FA	94.5 ± 1.4	96.8 ± 0.8	92.3 ± 1.4	97.4 ± 0.5	88.7 ± 3.2	91.8 ± 3.8	88.8 ± 1.2	93.5 ± 2.9

[a]Results are expressed as weight percent of total FA. Data are mean ± SD for several samples (n = 5–6).

as an energy storage site, and that MUFA, which is easily oxidized as an energy source, rather than DHA, which is hardly oxidized, is accumulated. The fluctuation of the DHA levels in TAG from pyloric cecum and other viscera between the samples may be influenced by variations in the FA composition of their prey lipids.

FA composition in tissue phospholipids. Similar to the FA composition of its tissue TAG, high levels of n-3 PUFA (20.3–49.0% for PE and 26.4–44.1% for PC) were found in the tissue phospholipids of both samples 1 and 2 (Tables 5 and 6). Four major FA were also found in the PE of all specimens at levels of about 5% or more of the TFA: two SFA, 16:0 (7.1–17.5%) and 18:0 (12.0–16.7%); one MUFA, 18:1n-9 (4.6–8.9%); and one n-3 PUFA, DHA (15.7–43.0%). Similar FA were also the major components in the PC: two SFA, 16:0 (25.2–34.6%) and 18:0 (2.1–6.1%); one MUFA, 18:1n-9 (5.1–10.4%); and two n-3 PUFA, EPA (6.9–12.4%) and DHA (17.4–34.9%). Noticeable amounts (>2%) of other FA were also found in these phospholipids: the MUFA 18:1n-7 (2.1–3.4% for PE and 1.0–2.7% for PC); the n-6 PUFA 20:4n-6 (4.8–8.8% for PE and 4.0–7.1% for PC); and the n-3 PUFA EPA (2.4–5.6% for PE). Although the major components of PE were similar to those of PC, slightly high n-3 PUFA levels in PC were found in all organs compared with those of PE. In particular, EPA was considered to be a major component of PC only.

It is well known that high levels of n-3 PUFA are generally found in the tissue PL of all marine fish species (1,44,45) because polar tissue PL work as cell membrane lipids and are generally rich in n-3 PUFA for membrane fluidity. Almost all DHA levels in the PL (PE and PC) in the *S. australasicus* lipids exceeded 20% of TFA, similar to other fish species including tuna species (1,44,45).

The major components in the PE were similar to those in the PC, and n-3 PUFA levels in PC were higher than those in PE; this is general tendency of all marine fish species. EPA was a major component of the PC, along with high levels of DHA.

Characteristically high levels of DHA in S. australasicus *muscle lipids.* Generally, it has been believed that only large-sized tuna species contain high levels of DHA in their tissues, whereas those in scomber species are comparatively low. Actually, *S. japonicus* and *S. commerson* contained comparatively low levels of DHA in their muscle TL, though body size, feeding habitat, and position in the marine grazing food chain of these fishes would be very similar to those of *S. australasicus*. One of the large differences in ecology is their migration; both *S. japonicus* and *S. commerson* migrate in coastal water, whereas *S. australasicus* migrates in offshore current, as tuna species do (22–26,35).

The occurrence of high levels of DHA in the organs of highly migratory tuna species is believed to be due to DHA

accumulation during long-distance migration (7,20), because MUFA and SFA are easily metabolized as energy sources prior to PUFA such as DHA (21). It is probably due to the requirement of higher energy usage of the fish during active migration in offshore current than coastal one need. Such offshore migration associated with FA metabolism might cause selective consumption of MUFA and SFA prior to PUFA, and that result in high levels of DHA in active migratory *S. australasicus* or in tuna species.

These previous findings (7,20,21) and our study may suggest that DHA is accumulated in neutral lipids of active fishes, as well as in neutral lipids of metabolically active organs. Actually, in the case of *Trachurus japonicus*, PUFAs were decreased prior to SFA and MUFA in the muscle TAG when the fish was non-active (30).

Based on the findings of the present study, it was inferred that high levels of DHA particularly in muscle TAG of *S. australasicus* may be caused by their offshore migration, similar to the situation in large-sized tuna species.

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Lipid Class and Fatty Acid Composition of the Boreal Soft Coral *Gersemia rubiformis*

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ABSTRACT: Total lipid, phospholipid, and FA composition and distribution of FA between polar lipids (PL) and neutral lipids (NL) were investigated in the boreal soft coral *Gersemia rubiformis* from the Bering Sea. The total lipids were mostly hydrocarbons and waxes (33.7%) and PL (33.1%). The content of monoalkyldiacylglycerols (9.7%) exceeded the content of TAG (6.7%). PC and PE constituted 31.4% and 25.6% of total phospholipids, respectively. Principal FA were 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 20:1n-7, 20:4n-6, 20:4n-3, 20:5n-3, 22:5n-3, 22:6n-3, 24:5n-6, and 24:6n-3. Most n-6 PUFA (52% of total FA) were associated with the PL fraction; this was especially true for arachidonic and tetracosapentaenoic acids. The NL were enriched with mono-, di-, trienoic, and n-3 PUFA. The variation in EPA levels in both NL and PL suggests an origin of this acid from lipids of diatoms consumed by the corals.

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A number of recently published articles testify to an increased interest of biochemists in lipids and FA of corals. Total lipid concentrations of 10–40% of dry biomass have been reported for a number of corals from tropical seas (1–3). Except for structural functions in cell membranes, the main role of coral lipids is to serve as a long-term energy reserve (4). In particular, it has been shown that some species of reef-building corals contain enough lipids to support their normal caloric demand for up to 114 d when no photosynthetically fixed carbon is transported from symbiotic algae to the coral host under conditions of insufficient sunlight (5). Global coral bleaching events in tropical oceans that resulted in a large-scale coral mortality and degradation of coral reef communities are accompanied by significant variations in total lipid content and proportions of the main lipid classes in coral colonies (6). Also, corals experience a natural variation in lipid concentrations during an annual cycle (7). Corals are rich in PUFA that are converted to biologically highly active oxylipins (including prostaglandins, oxypolyunsaturated acids, and clavulones) in the tissues of several coral species (8). Many coral families are characterized by the presence of unusual FA, for example, tetracosapolyenoic acids, and it has been suggested that the FA composition may be useful for the biochemical classification of these coelenterates (9).

The overwhelming majority of investigations of lipid and FA compositions have been carried out for reef-building stony coral species from the warm Caribbean, Red Sea, Sea of Japan, and Hawaii (3,6,7,10,11). Noticeably fewer articles have been dedicated to the lipid composition of algal symbionts of hermatypic corals (12) and tropical soft corals (13–15). Only a few works on lipids of soft corals from cold-water seas have been published. Oxylipins derived from arachidonic acid have been studied in the soft coral *Gersemia fruticosa* from the White Sea (16), and the FA compositions of some Octacorallia species from the Sea of Okhotsk have been investigated (17). Earlier, we made an attempt to determine the oxylipin composition and seasonal variations in the main FA for the soft coral *Gersemia rubiformis* (18). This paper examines total lipid, lipid class, phospholipid, and FA compositions, as well as the distribution of FA between polar lipids (PL) and neutral lipids (NL) in the soft coral *G. rubiformis* from the Bering Sea.

EXPERIMENTAL PROCEDURES

Colonies of the soft coral *G. rubiformis* (class Octocorallia, order Alcyonaria, family Alcyoniidae) were collected by SCUBA divers in the Avachinskaya Guba Inlet (52°46’38’’N, 158°36’55’’E, Starichkov Island, the Bering Sea) in August 2004 at 12 m depth. The colonies were washed in seawater and carefully cleaned of all noncoral debris. For a lipid analysis, at least three different colonies were used.

Extraction of total lipids was conducted according to Bligh and Dyer (19). The total lipid content was determined by gravimetry after lipid extract evaporation under reduced pressure. Lipid classes were separated by one-dimensional silica gel TLC. The precoated Merck (Darmstadt, Germany) Kieselgel 60 G plates (10 cm × 10 cm) were first developed to their full length with $C_6H_6/Et_2O/CH_3COOH$ (70:30:1, by vol), and finally to 25%-length with $CHCl_3/CH_3OH/C_6H_6/28\%$ NH_4OH (65:30:10:6, by vol). After drying in a stream of air, plates were sprayed with 10% H_2SO_4/MeOH and heated at 180°C for 10 min. The chromatograms were scanned by an image scanner Epson Perfection 2400 PHOTO (Nagano, Japan) in a grayscale mode. The software used for scanning was Adobe Photoshop (Adobe Systems, San Jose, CA). Percentages of lipid contents were determined based on band intensity with the use of the

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Abbreviations: CAEP, ceramide aminoethylphosphonate; MADAG, monoalkyldiacylglycerol; NL, neutral lipids; PL, polar lipids.

image analysis program Sorbfil TLC Videodensitometer DV (Krasnodar, Russia). The units were calibrated using known standards for each lipid class.

The total lipids were separated into NL and PL fractions by column chromatography on silica gel according to Rouser *et al*. (20). Polar lipids were separated by two-dimensional silica gel TLC with $CHCl_3/CH_3OH/C_6H_6/28\%$ NH_4OH (65:30:10:6, by vol) in the first direction and $CHCl_3/CH_3OH/CH_3COOH/CH_3COCH_3/C_6H_6/H_2O$ (70:30:4:5:10:1, by vol) in the second direction. To identify lipids on TLC plates the same specific spray reagents used earlier were employed (21). The phospholipid content was determined spectrophotometrically after digestion with perchloric acid (22). FAME were obtained by a sequential treatment of the lipids with 1% MeONa/MeOH and 5% HCl/MeOH according to Carreau and Dubacq (23) and purified by preparative TLC development in benzene. *N*-Acylpyrrolidide derivatives of FA were prepared by direct treatment of the FAME with pyrrolidine/acetic acid (10:1, by vol) in a capped vial (24 h, 25°C) followed by ethereal extraction from the acidified solution and purification by preparative TLC developed in ethyl acetate.

GC analysis of FAME was carried out on a Shimadzu GC-17A chromatograph (Kyoto, Japan) with a flame ionization detector on a SUPELCOWAX 10 (Supelco, Bellefonte, PA) capillary column (25 m × 0.25 mm i.d.) at 210°C. Helium was used as the carrier gas. FAME were identified by a comparison with authentic standards and using a table of ECL (24). The structures of FA were confirmed by GC–MS of their methyl esters and *N*-acylpyrrolidide derivatives. GC–MS of FAME was performed with a Shimadzu GCMS-QP5050A instrument (Kyoto, Japan). A Supelco MDN-5S (Bellefonte, PA) capillary column (30 m × 0.25 mm i.d.) was used at 160°C with a 2°C/min ramp to 240°C, which was held for 20 min. Injector and detector temperatures were 250°C. GC–MS of *N*-acylpyrrolidides was performed on the same instrument; injector and detector temperatures were 300°C, and column temperature was 210°C with a 3°C/min ramp to 270°C, which was held for 40 min.

The data were assessed statistically by a one-way ANOVA and Tukey HSD-test for *a posteriori* comparisons. Differences with $P < 0.05$ were taken as significant. All tests were performed using Statistica 6.0 statistical software.

RESULTS AND DISCUSSION

Total lipids constituted 2.2% of the wet weight in *G. rubiformis*. The percentages of individual lipid classes and phospholipids are shown in Tables 1 and 2, respectively. Similar to many species of Coelenterates (2,3,25), the total lipids of *G. rubiformis* contained a considerable proportion of NL, in particular hydrocarbons and wax esters (Table 1). Intriguing was the presence of a remarkable amount (up to 12% of the total lipids) of a less common monoalkyldiacylglycerol (MADAG). The content of TAG was lower than that of MADAG (Table 1). The presence of MADAG (up to 17% of total lipids) was previously shown for some species of scleractinian, soft, and hydroid corals from Okinawan, Caribbean, and Red Sea regions (2,3,25). In the NL fraction of *G. rubiformis*, sterols, FFA, and DAG constituted 30%. The content of the sterols detected in the total lipids of *G. rubiformis* did not appreciably exceed an average amount of the sterol fraction (about 7%) reported earlier for several species of hermatypic (6) and soft corals (3). In our opinion, the noticeable level of FFA detected in *G. rubiformis* (Table 1), as well as the extremely high level (up to 22% of total lipids) of FFA described by Grottoli *et al*. (6) for some reef-building coral species, may be a result of high phospholipase activity in these organisms, but this supposition needs further investigation. Until now, the content of DAG in corals was not clearly documented. Our data on the DAG content in *G. rubiformis* (2.9%) noticeably differed, for example, from similar analyses reported for *Porites compressa* and *Montipora verrucosa* in which the percentages of DAG were below the detection limit and amounted to 30%, correspondingly (6). Similarity of the total NL profile of *G. rubiformis* to those of other coral species makes it possible to postulate that the DAG content depends mainly on environmental conditions and cannot serve as a taxonomic characteristic of an individual coral species.

In *G. rubiformis*, PL concentrations were 31% of total lipids (Table 1). Most of the PL (more than 80%) was composed of phospholipids. The main phospholipids identified were PC, PE, and PS (Table 2). Along with the widespread phospholipids (PC, PE, and PS), two types of less common phosphonolipids, ceramide aminoethylphosphonate (CAEP) and ceramide methylaminoethylphosphonate, constituting 29% of phosphorus-containing lipids, were identified (Table 2). Noticeable was a distinct lack of phospholipid lyso-forms in *G. rubiformis*. In 1979, PC, PE, PS, and CAEP were reviewed by Joseph (25) as the principal phospholipids for several Anthozoa species. A similar phospholipid distribution was observed later in 3 species of tropical gorgonians (*Psam-*

TABLE 1
Content of Individual Lipid Classes in *Gersemia rubiformis*[a]

Lipid class	Content (% total lipids)
Waxes and hydrocarbons	29.5 ± 4.9
MADAG	9.7 ± 2.8
TAG	6.7 ± 1.9
FA	8.5 ± 1.8
DAG	2.9 ± 0.7
Sterols	8.9 ± 1.6
PL	31.1 ± 5.0

[a]Data, presented as mean ± SD, $n = 5$. MADAG, monoalkyldiacylglycerol; PL, polar lipids.

TABLE 2
Phospholipid Composition of *Gersemia rubiformis*[a]

Phospholipid	Content (% total lipids)
PC	31.4 ± 6.2
PE	25.6 ± 1.5
PS	14.1 ± 3.2
Ceramide aminoethylphosphonate	15.6 ± 1.4
Ceramide methylaminoethylphosphonate	13.3 ± 3.0

[a]Data presented as mean ± SD, $n = 5$.

mogorgia nodosa, *Bebryce indica*, and *Mopsella aurantia*) (26) and in 22 species of tropical alcyonarians, belonging to the genera *Sinularia*, *Lobophytum*, and *Sarcophyton* (15). PC, PE, PS, and CAEP amounted to up to 40.4, 30.4, 19.8, and 23.1%, correspondingly, of the total phospholipid fraction of the soft coral mentioned above (15,26). The main phospholipids from 11 species of tropical gorgonians, belonging to the genera *Gorgonia*, *Pseudopterogorgia*, and *Eunicea*, were characterized as PE, PC, and PS by Carballeira (13), but either the percentages of phospholipids or the presence of CAEP were not recorded.

In tropical soft corals, about three-fourths of PE and one-quarter of PC and PS molecules have been shown to be present in plasmalogenic forms (15); the same phenomenon was observed for the main phospholipid classes in the boreal soft coral *G. rubiformis* (data not shown). Both plasmalogens and phosphonolipids appeared to be common in soft corals, and have chemical bonds stable against hydrolytic enzymes such as phospholipases and phosphatases. Hydrolysis of MADAG by lipases is also hindered. We agree with Joseph (25) that the unusual plasmalogens, phosphonolipids, and MADAG may stabilize coral membranes that are exposed to highly active hydrolytic enzymes.

Within the total lipids of *G. rubiformis*, the principal FA were 16:0, 18:1n-9, 20:1n-7, 20:4n-6, 20:5n-3, 24:5n-6, and 24:6n-3, as shown in Table 3. In comparison with 23 FA described previously (18), we defined the FA composition of *G. rubiformis* more exactly (46 FA) and have redefined the earlier assumed tetracosateraenoic acid 24:4 as tetracosapentaenoic acid 24:5n-6. The mass spectra of methyl ester of this acid gave a molecular ion peak at *m*/*z* 372 (M^+), confirming the presence of five double bonds and a C_{24} chain in the original FA, and a strong peak at *m*/*z* 108, which is characteristic of the mass spectra of methyl esters of methylene-interrupted PUFA of the n-3 family. In contrast, in the n-6 family, a strong peak at *m*/*z* 150 is particularly abundant (27). The mass spectra of the *N*-acylpyrrolidide derivative of 24:5n-6 gave molecular ion peaks at *m*/*z* 411 (M^+) and five pairs of fragments at *m*/*z* 154 and *m*/*z* 166, *m*/*z* 194 and *m*/*z* 206, *m*/*z* 234 and *m*/*z* 246, *m*/*z* 274 and *m*/*z* 286, and *m*/*z* 314 and *m*/*z* 326, indicating that the double bonds were localized at the 6, 9, 12, 15, and 18 carbon atoms of the original FA (17,28).

PUFA with 24 carbon atoms and 5 to 6 methylene-interrupted *cis*-double bonds are typical constituents of representatives of all orders of subclass Octacorallia (Anthozoa): Alcyonaria, Gorgonaria, Helioporida, and Pennatularia (9,13). A non-food origin of tetracosapolyenoic acids in Octacorallia is strongly supposed (9). Thus, the large amount of the tetracosapolyenoic acids (10.4% of total FA) defined in the boreal soft coral *G. rubiformis* confirms chemotaxonomic significance of these acids for the octacorals.

Saturated, monoenoic, and polyunsaturated FA accounted, correspondingly, for 10.6, 25.0, and 61.8%, of the total FA composition of *G. rubiformis* collected in August (Table 3) at the highest (8–10°C) water temperature in the Avachinskaya Guba Inlet. We cannot assert that a noticeable amount of unsaturated FA is a sign of boreal octacorals inhabiting perennial cold waters, because no remarkable variation was noted for the concentrations of unsaturated FA in *G. rubiformis* dur-

TABLE 3
FA Composition of Total (TL), Neutral (NL), and Polar Lipids (PL) of *Gersemia rubiformis*[a] FA

	TL (n = 6)	NL (n = 4)	PL (n = 4)
14:0**	1.0 ± 0.4	1.4 ± 0.1	0.6 ± 0.1
i-15:0	0.2 ± 0.1	0.1 ±0.0	—
15:0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0
15:1	0.2 ± 0.1	0.1 ± 0.0	0.6 ± 0.5
16:0	7.4 ± 2.2	10.4 ± 1.9	5.5 ± 2.1
16:1n-9**	0.5 ± 0.2	0.6 ± 0.0	0.3 ± 0.0
16:1n-7**	2.9 ± 0.8	3.8 ± 0.0	1.2 ± 0.3
16:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
i-17:0	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
16:2*	0.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.0
br-17:1**	0.7 ± 0.1	1.3 ± 0.3	0.4 ± 0.2
16:2*	0.5 ± 0.1	1.0 ± 0.3	0.4 ± 0.0
17:0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
17:1n-9	0.9 ± 0.8	0.2 ± 0.2	0.4 ± 0.2
i-18:0**	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
br-18:1	0.1 ± 0.1	0.3 ± 0.0	—
18:0	1.4 ± 0.5	1.4 ± 0.4	1.3 ± 0.3
18:1**	0.2 ± 0.2	0.3 ± 0.0	0.1 ± 0.0
18:1n-9**	3.8 ± 1.0	5.8 ± 1.3	1.5 ± 0.2
18:1n-7**	3.0 ± 0.9	3.5 ± 0.2	2.0 ± 0.1
18:2n-6*	1.0 ± 0.3	2.0 ± 0.7	0.4 ± 0.1
br-19:1**	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
19:1	0.2 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
18:3n-3*	0.3 ± 0.1	0.7 ± 0.3	0.1 ± 0.0
19:2+18:4n-3**	0.8 ± 0.2	1.4 ± 0.4	0.2 ± 0.1
20:0	0.1 ± 0.0	0.1 ± 0.0	—
20:1n-11**	0.8 ± 0.2	1.1 ± 0.1	0.4 ± 0.1
20:1n-9*	0.9 ± 0.1	1.4 ± 0.6	0.4 ± 0.1
20:1n-7	9.5 ± 1.4	5.7 ± 0.4	7.2 ± 2.7
5,11-20:2*	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.0
20:2n-6	0.6 ± 0.1	0.8 ± 0.2	0.4 ± 0.1
20:3n-9**	0.6 ± 0.2	0.6 ± 0.1	0.2 ± 0.0
20:4n-6**	22.8 ± 4.7	10.8 ± 0.3	39.1 ± 3.2
20:3n-3*	0.4 ± 0.2	0.5 ± 0.1	0.2 ± 0.0
20:4n-3**	1.5 ± 0.7	2.2 ± 0.3	0.3 ± 0.1
20:5n-3	16.5 ± 3.4	23.1 ± 6.4	13.6 ± 3.9
22:1n-11	0.6 ± 0.1	0.7 ± 0.2	0.3 ± 0.1
22:1	0.2 ± 0.1	0.4 ± 0.1	—
22:2n-3	0.6 ± 0.2	0.8 ± 0.0	0.3 ± 0.1
22:4n-6	0.9 ± 0.4	0.4 ± 0.0	1.2 ± 0.6
22:5n-6	0.2 ± 0.1	0.3 ±	0.4 ± 0.2
22:4n-3	0.3 ± 0.2	0.4 ± 0.1	—
22:5n-3**	1.6 ± 1.1	1.7 ± 0.2	0.5 ± 0.3
22:6n-3	2.0 ± 0.3	3.0 ± 0.0	2.4 ± 0.7
24:5n-6**	5.9 ± 1.3	2.2 ± 0.2	11.4 ± 2.1
24:6n-3	4.4 ± 1.2	4.3 ± 0.5	4.3 ± 1.8
Saturated	10.6 ± 3.3	14.1 ± 2.7	8.0 ± 2.6
Monoenoic**	25.0 ± 2.4	25.3 ± 0.4	14.9 ± 2.8
Dienoic**	3.7 ± 0.8	6.0 ± 1.2	1.8 ± 0.1
Trienoic*	1.4 ± 0.4	1.8 ± 0.5	0.4 ± 0.1
Tetraenoic**	31.4 ± 5.7	15.9 ± 0.3	52.0 ± 3.5
Pentaenoic	18.9 ± 3.7	25.9 ± 5.9	14.7 ± 4.2
Hexaenoic	6.4 ± 0.9	7.3 ± 0.5	6.7 ± 1.2
n-6**	31.5 ± 5.9	16.3 ± 1.1	52.9 ± 3.6
n-3*	27.1 ± 5.0	35.9 ± 4.9	21.4 ± 5.4

[a]Values are reported as percent of total FA, mean value ± SD. In PL and NL fractions, FA marked by * and ** have significantly different contents at $P < 0.05$ and $P < 0.01$, correspondingly.

ing the annual cycle (18), and a high concentration of unsaturated FA has been documented for some tropical octacoral species (e.g., 74.7% for *Mopsella aurantia* (14)).

The FA composition of NL and PL fractions in *G. rubiformis* was investigated (Table 3). The concentration of mono-, di-, and trienoic acids in NL was significantly higher ($P < 0.05$) than that for PL. No significant difference between the NL and PL fractions was noted for the concentrations of saturated, penta-, and hexaenoic acids ($P > 0.05$). The NL contained much lower concentrations of the tetraenoic acids, were enriched with EPA (20:5n-3), and were deficient in arachidonic (20:4n-6), docosatetraenoic (22:4n-6), and tetracosapentaenoic (24:5n-6) acids. Accordingly, the fraction of n-3 FA was concentrated in the NL, and the fraction of n-6 FA significantly dominated (n-6/n-3 = 2.6) in the PL ($P < 0.01$). A similar predomination of the n-6 PUFA in phospholipid fractions from 11 species of tropical gorgonian octacorals was reported by Carballeira (13), whereas in the hermatypic hexacoral *Stylopora pistillata*, the ratio n-6/n-3 in PL FA was about 1.0 (11).

Based on the biosynthesis of n-6 FA from linoleic acid (18:2n-6) and n-3 FA from linolenic acid (18:3n-3), it is suggested that 24:5n-6 and 24:6n-3 acids may originate through elongation-desaturation of arachidonic acid (20:4n-6) and EPA (20:5n-3), respectively (17). PL and NL are often called structural and storage (reserve) lipids according to their main functions in living cells; furthermore, an excess of lipids and FA obtained from food is accumulated by animals mainly in storage NL. The predominance of the n-6 C_{20}–C_{24} PUFA in structural lipids of *G. rubiformis* indicates that these n-6 FA are basically synthesized by octacoral host tissues, whereas the predominance of EPA (20:5n-3) in the storage lipids of *G. rubiformis* and a relatively high variation in the 20:5n-3 content between individual coral colonies (Table 3) is most likely caused by the variability in the incoming main part of 20:5n-3 from food sources.

Similar to all heterotrophic soft corals, one of the important food sources for *G. rubiformis* is phytoplankton. In the Avachinskaya Guba Inlet, where the coral originated, the phytoplankton consists of two main groups of microalgae, diatoms, and phytoflagellates (mainly, dinoflagellates) (29). Diatoms are characterized by the presence of high level of 20:5n-3, and two other FA, 18:4n-3 and 22:6 n-3, are considered to be the biomarkers of dinoflagellates (30). It is well known that some distinctive FA from foods may accumulate in the lipids of consumers as biomarker FA. We compared our data on seasonal changes of FA composition in *G. rubiformis* obtained earlier (18) with the seasonal dynamics of phytoplankton population density in the Avachinskaya Guba Inlet (29). In *G. rubiformis*, the maximum content of 18:3n-3 and 18:4n-3 (up to 17% of total FA) was observed in April and November; the maximum content of 20:5n-3 and the lowest of 18:4n-3 were detected in June (18). According to Konovalova (30), two abundant blooms of the dinoflagellates constituting more than 90% of phytoplankton biomass appeared in late April and October. In June and August, the proportion of the dinoflagellates in phytoplankton decreased up to 55% owing to an increase on the part of the diatoms. Thus, we observe a simultaneous increase in the biomass of a certain phytoplankton group and in the concentration of coral FA that are the recognized biomarkers of the phytoplankton group. It can be assumed that a high level of 18:3n-3, 18:4n-3 (in sum, up to 28% of total FA), and 22:6 n-3 (about 7% of total FA) found in phospholipids of some tropical gorgonians (13) is caused by feeding of the corals on certain phytoplankton (e.g., dinoflagellates) containing high amounts of these FA.

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METHOD

The Analysis of Lipids *via* HPLC with a Charged Aerosol Detector

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ABSTRACT: Because most lipid extracts are a mixture of saturated and unsaturated molecules, the most successful strategies for the quantitative analysis of lipids have involved the use of so-called "mass" or universal detectors such as flame ionization detectors and evaporative light scattering detectors. Recently a new type of HPLC "mass" detector, a charged aerosol detector (CAD), was developed and is now commercially available. This detection method involves nebulizing the HPLC column effluent, evaporating the solvents, charging the aerosol particles, and measuring the current from the charged aerosol flux. In the present study, the CAD was evaluated with several normal phase and reverse phase HPLC methods commonly used for the quantitative analysis of lipid classes and lipid molecular species. The CAD detected common lipids such as triacylglycerols, diacylglycerols, glycolipids, phospholipids, and sterols. Lower molecular weight lipids such as free FA had smaller peak areas (50–80% lower). FAME were not detected by the CAD, probably because they were completely evaporated and did not form aerosol particles. The minimum limits of detection of the CAD with lipids varied with different mobile phase solvents. Using solvent systems that were predominantly hexane, the minimum limits of detection of triacylglycerols, cholesterol esters, and free sterols were about 1 ng per injection and the mass-to-peak area ratio was nearly linear from the range of about 1 ng to about 20 mg per injection. Three other solvents commonly used for HPLC lipid analysis (methanol, isopropanol, and acetonitrile) caused higher levels of background noise and higher minimum limits of detection. These experiments indicate that the CAD has the potential to become a valuable tool for the quantitative HPLC analysis of lipids. Long-term studies are needed to evaluate full instrument performance.

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Several reviews have described in detail the numerous HPLC methods that have been developed for the quantitative analysis of plant, animal, and microbial lipids (1–4). The most common and least expensive detection method for HPLC is the UV-visible detector (1). UV-visible detectors have proven to be useful for those lipids that have chromophores, but they have been of limited use for the analysis of lipid extracts that contain a mixture of saturated and unsaturated molecules. Beginning in the mid-1980s, the first HPLC methods were developed for the quantitative analysis of lipids using "mass" detectors such as FID and ELSD, which could detect both saturated and unsaturated lipids. The first method that employed an ELSD for the analysis of lipid classes was published by W.W. Christie in 1985 (5). In 1990, our group published a similar HPLC method for the analysis of lipid classes using an FID (6) and demonstrated that FID and ELSD technologies were comparable. Although FIDs were successfully used for HPLC analysis for several years, FID technology was quickly surpassed by ELSD technology, and the manufacturing of FID ceased in the mid-1990s. In the last 15 years the sensitivity of ELSD has improved greatly and numerous methods have been developed for the analysis of lipids by HPLC-ELSD (1,3). Whereas the minimum limits of detection with early ELSD were about 10–20 μg per peak, the minimum limits of detection of lipids with modern ELSD has improved to about 50 to 100 ng (1).

Besides the FID and ELSD, a third type of aerosol-based detector was reported with examples of applications for lipid analysis (7). When used with a microbore column, this condensation nucleation light scattering detector (not commercially available) was reported to have minimum limits of detection of less than 1 ng.

Recently a new type of HPLC "mass" detector, a charged aerosol detector (CAD), was developed and is now commercially available (8,9). This detection method involves nebulizing the HPLC column effluent, evaporating the solvents, charging the aerosol particles, and measuring the current from the charged aerosol flux. In the present study, the CAD was evaluated with several normal phase and reverse phase HPLC systems commonly used for the quantitative analysis of lipid classes and lipid molecular species.

EXPERIMENTAL PROCEDURES

Materials. All organic solvents were freshly opened bottles of Baker Analyzed® HPLC grade solvents, obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ). Chromatographic standards of lipids [Non-polar Lipid Mix A (cat# 1129), Non-polar Lipid Mix B (cat# 1130), and Polar Lipid Mix (cat# 1127), all for TLC] were obtained from Matreya (Pleasant Gap, PA). Lecithin granules (97% soy phosphatides) were obtained from the Vitamin Shoppe (North Bergen, NJ). Barley lipid extract was obtained by extracting ground barley kernels (the cultivar was Doyce) with hexane, as previously described (10).

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Abbreviations: CAD, charged aerosol detector; T, tocopherol; T3, tocotrienol.

HPLC system. The HPLC was an Agilent Model 1100 with autosampler. For experiments that involved injecting various masses of lipids, solutions were prepared at concentrations of 0.001, 0.01, 0.1, and 1.0 mg/mL, and triplicate injections of 1, 2, 5, and 10 μL of each concentration were made. Detection was by two methods, in series. An Agilent Model 1100 Fluorescence Detector (Agilent Technologies, Avondale, PA), with excitation at 294 nm and emission at 326 nm (flow cell volume, 8 μL) was used upstream of an ESA Corona™ Charged Aerosol Detector (ESA Biosciences, Chelmsford, MA) operated with nitrogen as a nebulizing gas and at a range of 500 pA. All other CAD parameters were preset by the manufacturer. The volume of solvent in the 50 cm of 0.010-in. i.d. PEEK tubing between the fluorescence detector and CAD was approximately 25 μL.

Normal phase nonpolar lipid HPLC analyses. This method was originally developed for use with an evaporative light scattering detector (11). A LiChrosorb 7 μm diol column (3 × 100 mm, packed by Chrompack, Raritan, NJ) was used. The binary gradient had a constant flow rate of 0.5 mL/min, with Solvent A = 99.9 % hexane/0.1% acetic acid (all solvent compositions are vol/vol), Solvent B = 99% hexane/1% isopropanol. Gradient timetable: at 0 min, 100/0 (%A/%B); at 8 min, 100:0; at 10 min, 75:25; at 40 min, 75:25; at 41 min, 100:0; and at 60 min, 100:0. These nonpolar lipid components were identified by comparison to the retention times of commercial standards.

Normal phase polar lipid HPLC analyses. Polar lipids (including acylated steryl glucosides, steryl glucosides, and phospholipids) were quantitatively analyzed by a similar method developed for use with an ELSD (12). The polar lipid components were identified by comparison to the retention times of commercial standards. The diol column and flow rates were the same as above. For isocratic analyses the mobile phase consisted of 45.9% hexane/50% isopropanol/4% water/0.1% acetic acid. For gradient analyses, the ternary gradient consisted of: Solvent A = 99.9% hexane/0.1% acetic acid, Solvent B = 100% isopropanol, and Solvent C = 100% water. Gradient timetable: at 0 min, 90:10/0 (%A/%B/%C); at 30 min, 58:40:2; at 40 min, 45:50:5; at 50 min, 45:50:5; at 51 min, 50:50:0; at 52 min, 90:10:0; and at 60 min, 90:10:0.

Normal phase tocopherol and tocotrienol HPLC analyses. Tocopherols and tocotrienols were quantified by using a modified version of the previously published method (13). The diol column and flow rates were the same as above. The binary gradient consisted of: Solvent A = 98% hexane/2% methyl *t*-butyl ether and Solvent B = 100% isopropanol. Gradient timetable: at 0 min, 100:0 (%A/%B); at 40 min, 100:0; at 45 min, 95:5; A/B, at 50 min, 95:5; at 51 min, 100:0; and at 60 min, 100:0. The MS was used to aid in peak identification. Gelcap supplements of tocopherols (Bio E Gamma Plex, Soloray Inc., Park City, UT) and tocotrienols (Tocopherol Complex, Solgar, Leonia, NJ) were purchased at a local vitamin store and the following peaks were confirmed by LC-MS, performed with an Agilent 1100 MSD equipped with an Atmospheric Pressure Chemical Ionization interface operated in the positive mode (drying gas at 6.0 L/min, nebulizer pressure at 60 psi, drying gas temperature at 350ºC, vaporizer gas temperature at 325ºC, capillary voltage at 4,000 V, and corona current at 4.0 μA, and fragmentor at 80 V): αT (M + 1 = *m/z* 431.4), αT3 (M + 1 = *m/z* 425.3), βT and γT (M + 1 = *m/z* 416.3), δT3 and γT3 (M + 1 = *m/z* 411.2), δT (M + 1 = *m/z* 402.3), and δT3 (M + 1 = *m/z* 397.1), where T = tocopherol and T3 = tocotrienol.

Reverse phase lipid molecular species HPLC analyses. This new method was recently developed to separate molecular species of triacylglycerols and other nonpolar lipids. The column was a Prevail RP18 3 μm (150 × 2.1 mm), packed by Alltech Associates, Deerfield, IL). The binary gradient had a constant flow rate of 0.5 mL/min, with Solvent A = 49.8% methanol/47% acetonitrile/3% dichloromethane/0.2% acetic acid, Solvent B = 100% isopropanol. Gradient timetable: at 0 min, 100:0 (%A/%B); at 20 min, 95:5; at 40 min, 50:50; at 50 min, 50:50; at 51 min, 100:0; and at 60 min, 100:0. These nonpolar lipid components were identified by comparison to the retention times of commercial standards.

RESULTS AND DISCUSSION

The first HPLC method to be evaluated with the CAD was a normal phase gradient elution method developed to quantitatively analyze the major nonpolar lipid class components (phytosterol esters, triacylglycerols, free FA, and free phytosterols) in vegetable oils and hexane extracts (11). The baseline was relatively smooth with this system (Fig. 1A). A commercial mixture that contained equal masses of five nonpolar lipid classes (cholesterol:oleate, triolein, oleic acid, methyl:oleate, and cholesterol) was injected in this system (Fig. 1B and C). Detector response (peak areas) for cholesterol:oleate and triolein were similar whereas that of cholesterol and oleic acid were approximately 10% and 80% lower, respectively. Methyl:oleate was not detected by the CAD, presumably because this lower MW component was completely evaporated and did not form aerosol particles. Similar results were previously reported for the ELSD—methyl esters were partially evaporated at a detector temperature of 40°C and completely evaporated at higher temperatures (1). Similarly (with the ELSD), free FA were partially evaporated at detector temperatures of 40°C and 60°C and completely evaporated at higher temperatures (1). Unlike most ELSDs, the nebulizer temperature of the CAD is preset and not variable. With this HPLC method, the minimum limits of detection with the CAD were about 1 ng, and the mass-to-peak area ratio was nearly linear from the range of about 1 ng to 20 ng per injection (Fig. 1D). In other experiments the standard curve was extended up to 20 μg and the mass-to-peak area ratio continued to remain nearly linear (data not shown).

The second HPLC method to be evaluated with the CAD was a normal phase method developed to quantitatively analyze the major polar lipid class components (mainly glycolipids and phospholipids) in extracts of plant material extracted with polar solvents (12). This system was evaluated with both isocratic elution (Fig. 2) and gradient elution (Fig. 3). In our

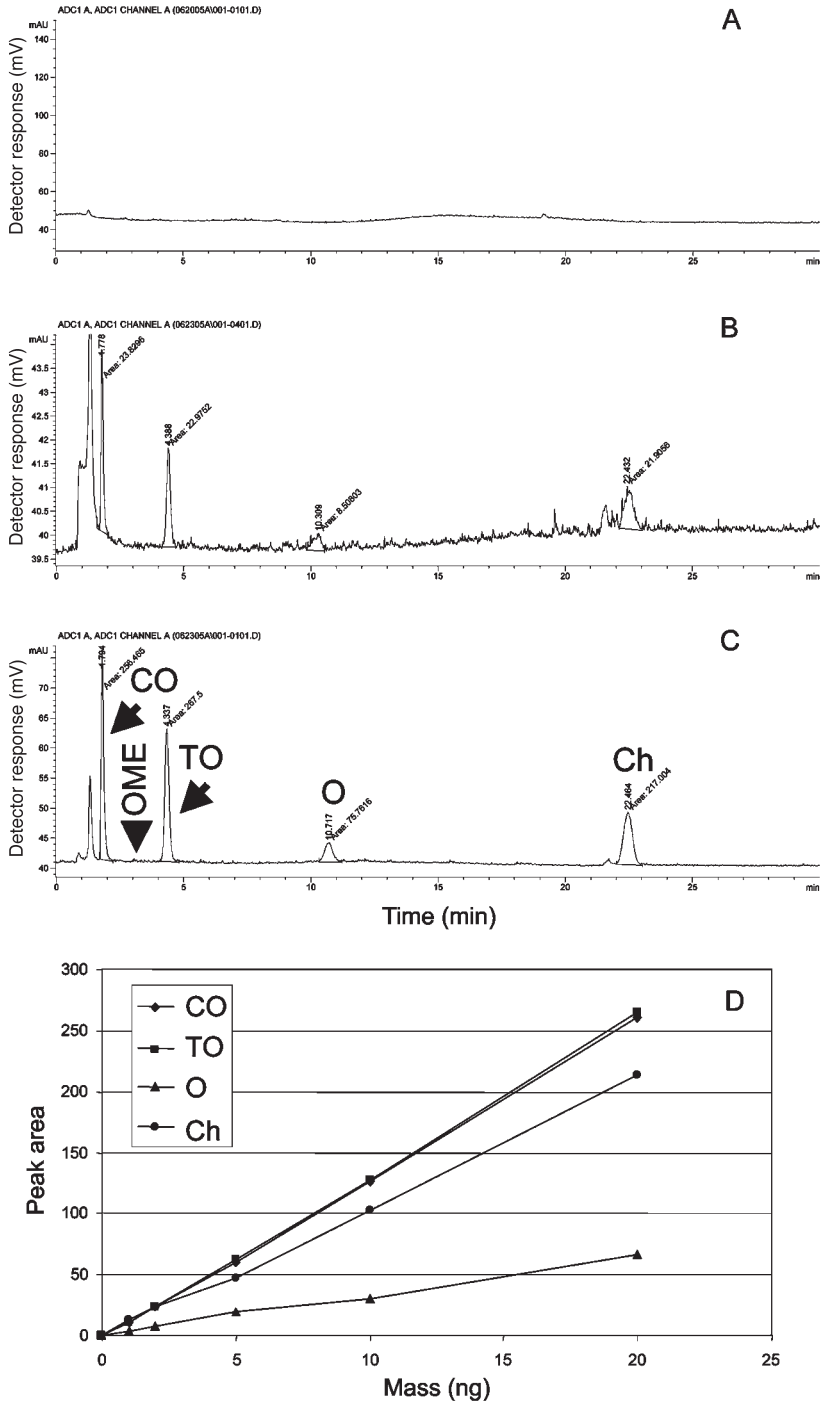

FIG. 1. Normal phase nonpolar lipid class HPLC analysis with detection via CAD. (A) Gradient blank, no lipid injected. (B) Matreya nonpolar lipid mix B with an injection comprised of 2 ng of each component. (C) Matreya nonpolar lipid mix B with an injection comprised of 20 ng of each component. (D) Mass versus peak area for Matreya nonpolar mix B. Abbreviations: CO, cholesteryl oleate; OME, oleate methyl ester; TO, triolein; O, oleic acid; Ch, cholesterol. Note that OME was totally evaporated and O was partially evaporated during detection and the other three lipids had similar detector response.

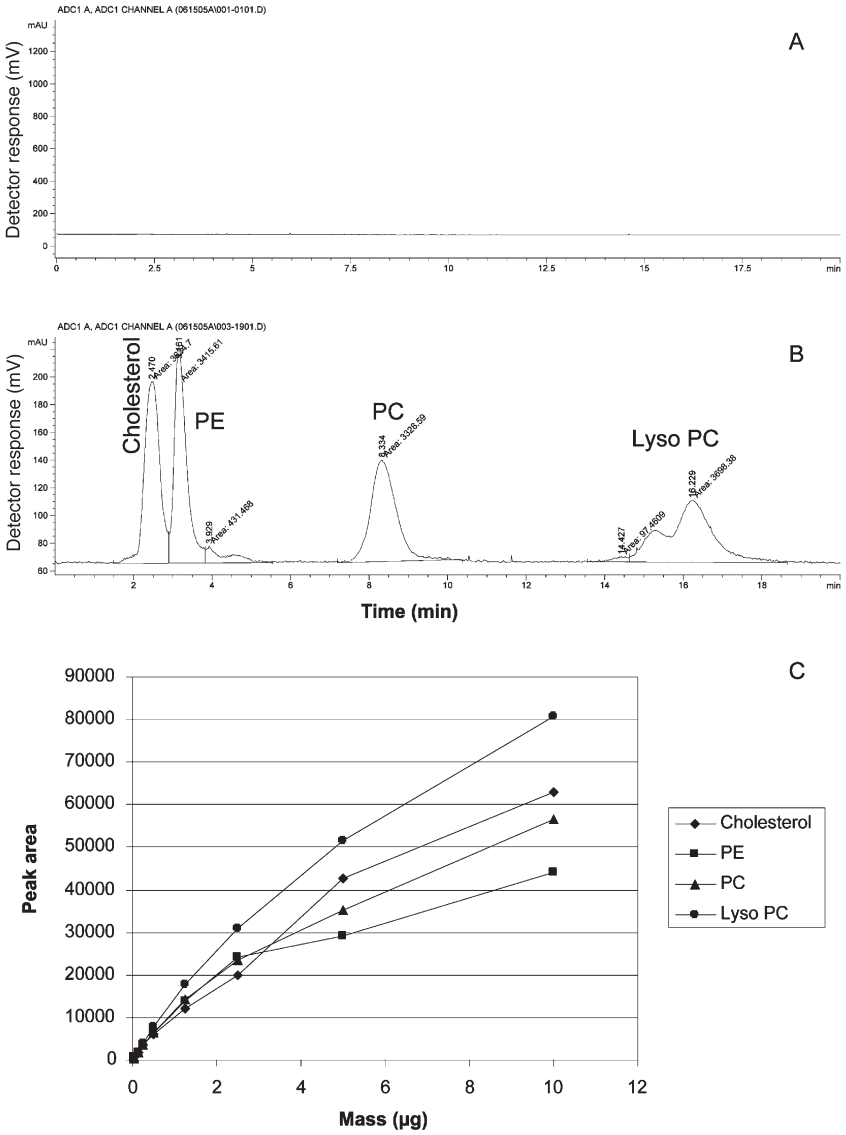

FIG. 2. Normal phase polar lipid class isocratic HPLC system. (A) Gradient blank, no lipid injected. (B) Matreya polar lipid mix with an injection comprised of 0.25 µg of each component. (C) Mass versus CAD peak area for Matreya polar lipid mix.

experience, the isocratic method is adequate to separate simple standard mixtures, but the gradient method is required to separate most plant lipid extracts. In the isocratic polar lipid system the baseline was relatively smooth (Fig. 2A). A commercial mixture that contained equal masses of four polar lipid classes (cholesterol, phosphatidylethanolamine [PE], phosphatidylcholine [PC], and lyso-phosphatidylcholine [lyso PC]) was injected in this system (Fig. 2B), and the components were well separated. With this HPLC system, the minimum limits of detection were about 25 ng, and the mass-to-peak area relationship was evaluated in the range from about 25 ng to about 10 µg per injection (Fig. 2C). Because it would have made the mass-to-peak area graph (Fig. 2C) difficult to read, the same data, with standard deviations are also reported in Table 1.

In using the normal phase polar lipid class gradient method, the baseline was relatively smooth until about 35 min, but it increased and "plateaued" from about 40 to 55 min, and then dropped back down to the original level for the remainder of the chromatogram (Fig. 3A). The four polar lipid classes in the standard were also well resolved in the gradient system (Fig. 3B). A sample of soy lecithin was then injected (Fig. 3C) and the CAD detected the same major glycolipids (acylated steryl glycoside and steryl glucoside) and phospholipids (PE, PC, lyso PC, and PI) that we previously observed by using an evaporative light scattering detector (14).

The next HPLC method evaluated was a normal phase method developed to separate the eight natural isomers of tocopherols and tocotrienols (13). Because tocopherols and to-

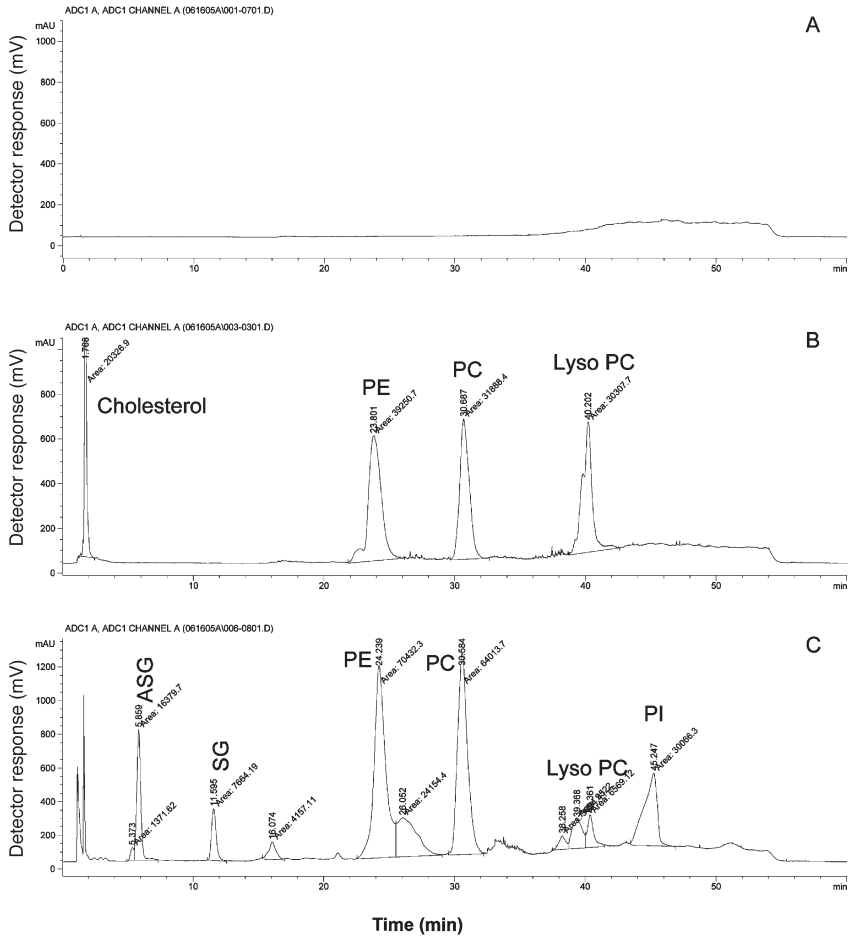

FIG. 3. Normal phase gradient chromatogram showing the separation and detector response of polar lipid standards. (A) Gradient blank, no lipid injected. (B) Matreya polar lipid mix with an injection comprised of 2.5 μg of each component. (C) Sample of soy lecithin, 20 μg of total lipid injected. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; Lyso PC, lysophosphatidylcholine, ASG, acylated steryl glycoside; SG, steryl glucoside; PI, phosphatidylinositol.

TABLE 1
Response of CAD with Polar Lipid Standards with the Isocratic HPLC Method with a Diol Column and a Mobile Phase of 45.45% Hexane/50.00% Isopropanol/4.50% Water/0.05% Acetic Acid by Volume[a]

Injected (μg)	Cholesterol (peak area ± SD)	PE (peak area ± SD)	PC (peak area ± SD)	Lyso PC (peak area ± SD)
0.025	735 ± 311	397 ± 74	408 ± 65	438 ± 108
0.05	669 ± 65	724 ± 48	756 ± 37	644 ± 158
0.125	1,753 ± 95	1,804 ± 53	1,722 ± 90	1,880 ± 129
0.250	3,647 ± 29	3,410 ± 31	3,381 ± 100	3,762 ± 182
0.5	5,986 ± 347	6,375 ± 126	6,418 ± 151	7,786 ± 261
1.25	12,070 ± 174	13,820 ± 37	14,120 ± 449	17,620 ± 254
2.5	19,920 ± 268	24,160 ± 194	23,420 ± 428	30,790 ± 334
5.0	42,750 ± 490	29,120 ± 409	35,370 ± 682	51,570 ± 1482
10.0	63,000 ± 184	44,040 ± 1980	56,480 ± 2247	80,920 ± 3285

[a]The values for mean and SD were from three injections of each sample.

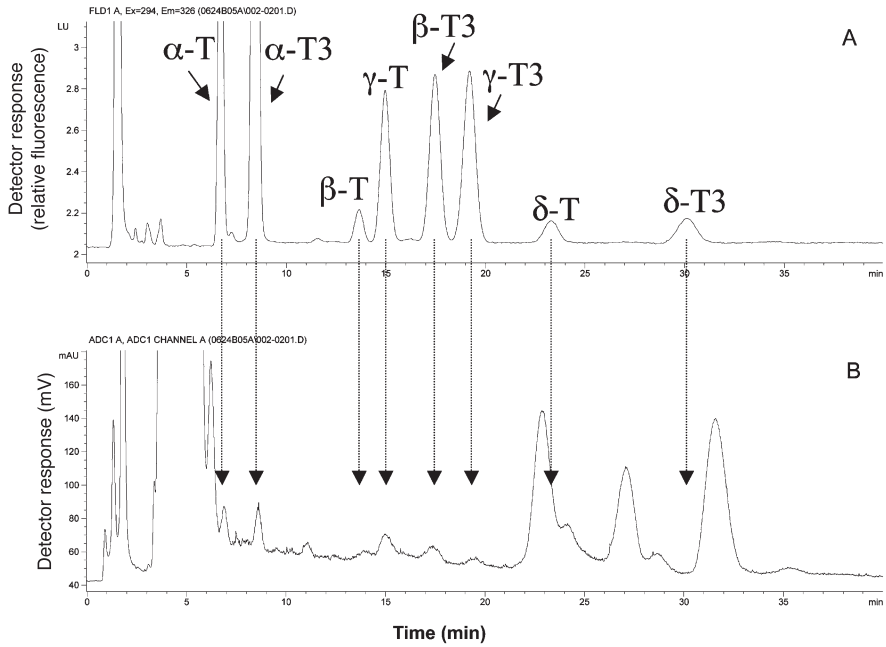

FIG. 4. Normal phase tocopherol/tocotrienol HPLC system showing the peaks of tocopherols and tocotrienols in unrefined oil obtained by extracting ground Doyce hulless barley kernels with hexane. (A) Detection was with a fluorescence detector (294 nm excitation and 326 nm emission). Abbreviations: T, tocopherol; T3, tocotrienol. (B) Detection with CAD.

cotrienols fluoresce, fluorescence detection (294 nm excitation and 326 nm emission) is a very selective detection method that has been used to accurately quantify these compounds, with minimum limits of detection of about 1 ng (15). Because the CAD also potentially has minimum limits of detection at about 1 ng, we prepared an extract of barley and compared the detection with the fluorescence detector (Fig. 4A) and the CAD (Fig. 4B). Interestingly, the CAD revealed several large peaks with retention times similar to tocopherols and tocotrienols (but since the components in these peaks did not fluoresce, they were not tocopherols or tocotrienols). Although it appears that the CAD could potentially be used to quantify some tocopherols and tocotrienols, the presence of other unknown peaks with similar retention times complicates the chromatogram. Under these conditions, it appears the fluorescence detection is still the HPLC detection method of choice for the quantitative analysis of tocopherols and tocotrienols.

The final HPLC method studied was a reverse phase method recently developed to separate the molecular species of triacylglycerols (e.g., triolein and trilinolein) and other nonpolar lipids. The baseline was noisier with this reverse phase system than with the normal phase systems (Fig. 5A). The reason for this increased noise was not examined. The nonpolar lipids in two commercial standard kits were separated with this system, at component concentrations of 25 ng (Fig. 5B) and 200 ng (Fig. 5C). Because the system was quite noisy, the minimum limits of detection with this system appear to be about 25 ng.

While conducting the previous experiments it was observed that the CAD baseline was relatively smooth with some solvents and noisy with others. The next experiment was designed to measure the effect of various common HPLC solvents on the CAD baseline (Fig. 6) at a constant flow rate of 0.5 mL/min, without a column and without injecting any lipid samples. The HPLC pump was programmed to deliver hexane for 10 min and then perform a 1-min gradient to transition to 100% isopropanol for the next 9 min, and similarly transition to methanol, water, isopropanol, and finally return to hexane. Among the four solvents evaluated in the first experiment, methanol produced the highest CAD background (Fig. 6A). Among the four solvents evaluated in the second experiment, acetonitrile produced the highest CAD background (Fig. 6B). Methanol and acetonitrile are the most common HPLC solvents for reverse phase HPLC. Clearly, more studies are needed to fully investigate the effects of these solvents on the noise and performance of the CAD.

These preliminary results indicate that the CAD has the potential to become a valuable tool for the quantitative HPLC analysis of lipids. The major advantages of the CAD are its low minimum limits of detection and its nearly linear mass-to-peak area relationship for many types of lipids. Long-term studies are needed to confirm that the results are reproducible and that the instrument is durable and reliable.

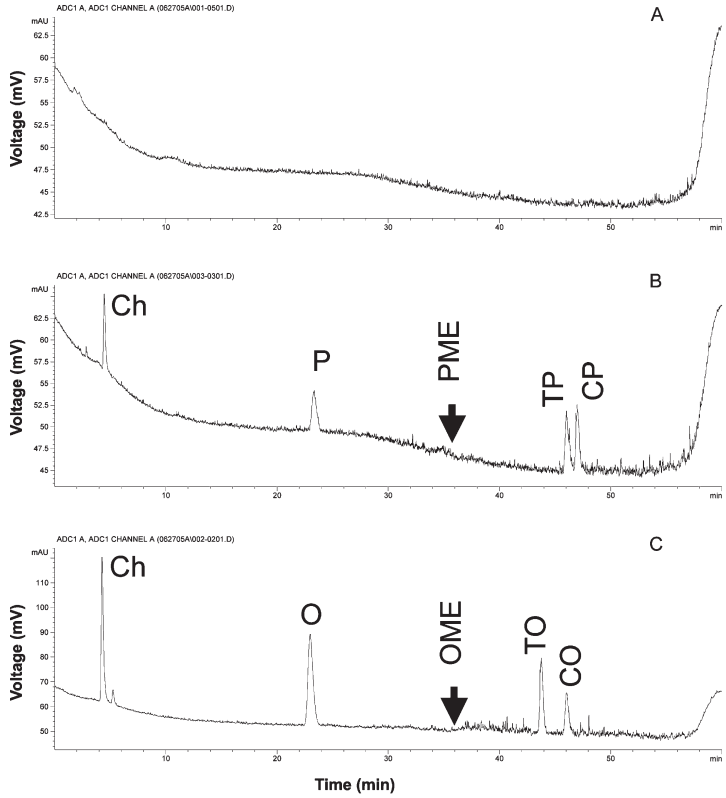

FIG. 5. Reverse phase nonpolar lipid HPLC system evaluated with Matreya nonpolar mix A and B. (A) Gradient blank, no lipid injected. (B) Matreya nonpolar lipid mix A with an injection comprised of 25 ng of each component. (C) Matreya nonpolar lipid mix B with an injection comprised of 200 ng of each component. Abbreviations: CP, cholesteryl palmitate; PME, palmitate methyl ester; TP, tripalmitin; P, palmitic acid; Ch, cholesterol. For other abbreviations, see Figure 1.

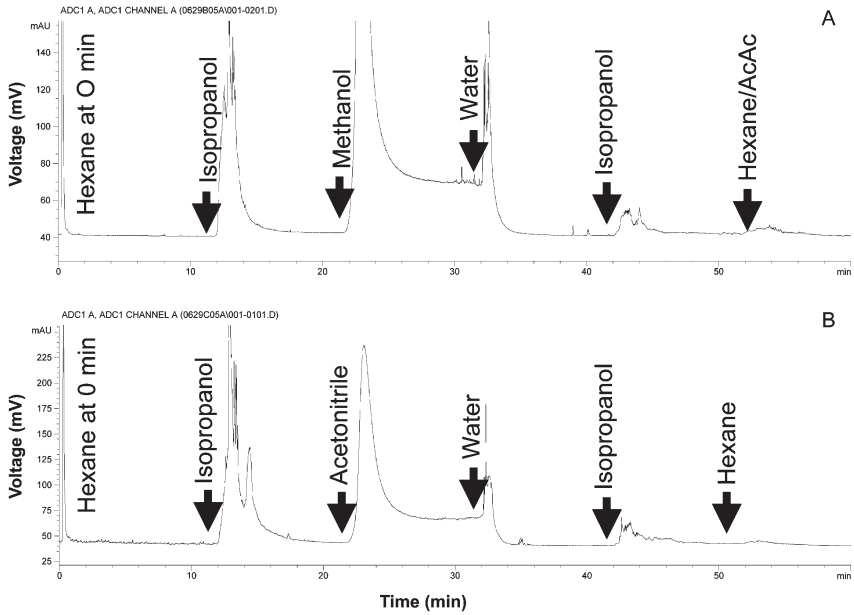

FIG. 6. The effect of various solvents on the CAD response with no HPLC column and no lipids injected. (A) Effect of hexane, isopropanol, methanol, and water (at a flow rate of 0.5 mL/min). (B) Effect of hexane, isopropanol, acetonitrile, and water (at a flow rate of 0.5 mL/min).

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Fatty Acid Production in *Schizochytrium* sp.: Involvement of a Polyunsaturated Fatty Acid Synthase and a Type I Fatty Acid Synthase

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ABSTRACT: *Schizochytrium* sp. is a marine microalga that has been developed as a commercial source for docosahexaenoic acid (DHA, C22:6 ω-3), enriched biomass, and oil. Previous work suggested that the DHA, as well as docosapentaenoic acid (DPA, C22:5 ω-6), that accumulate in *Schizochytrium* are products of a multi-subunit polyunsaturated fatty acid (PUFA) synthase (1). Here we show data to support this view and also provide information on other aspects of fatty acid synthesis in this organism. Three genes encoding subunits of the PUFA synthase were isolated from genomic DNA and expressed in *E. coli* along with an essential accessory gene encoding a phosphopantetheinyl transferase (PPTase). The resulting transformants accumulated both DHA and DPA. The ratio of DHA to DPA was approximately the same as that observed in *Schizochytrium*. Treatment of *Schizochytrium* cells with certain levels of cerulenin resulted in inhibition of ^{14}C acetate incorporation into short chain fatty acids without affecting labeling of PUFAs, indicating distinct biosynthetic pathways. A single large gene encoding the presumed short chain fatty acid synthase (FAS) was cloned and sequenced. Based on sequence homology and domain organization, the *Schizochytrium* FAS resembles a fusion of fungal FAS β and α subunits.

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DHA, along with other long chain (LC-, those fatty acids with carbon chain lengths greater than 18) PUFAs, have been identified as important dietary compounds for humans. The ω-3 LC-PUFAs, such as DHA and eicosapentaenoic acid (EPA, C20:5), are implicated in early neural and retinal development, the prevention of arteriosclerosis and coronary heart disease, alleviation of inflammation, and retarding the growth of tumor cells (2). The effects are seen as a result of the ω-3 fatty acids acting as competitive inhibitors of ω-6 fatty acids and from the beneficial effects of the ω-3 compounds in their own right (*3*).

A major dietary source of ω-3 LC-PUFAs is from marine fish or fish oil supplements. However, due to emerging concerns with sustainability of marine resources and with the levels of environmental contaminants (PCBs, dioxins, and mercury) in fish (4), major efforts have been made to identify or create alternative sources. One area that has received considerable attention is the creation of crop plants whose seed oils are enriched in LC-PUFA via the use of genetic engineering techniques (5, 6). The oils of these plants do not normally contain LC-PUFA but can contain significant amounts of the C18 PUFAs: linoleic (C18:2 ω-6) and α-linolenic (C18:3 ω-3) fatty acids. Most of the engineering strategies are based on modification of the plant's endogenously produced PUFA through introduction of foreign genes, encoding a variety of enzymes that can either elongate the fatty acid chain or insert additional *cis*-double bonds (i.e., elongases and desaturases, respectively). These efforts have achieved significant progress. Genes have been isolated that encode the entire suite of enzymes needed to convert linoleic and/or α-linolenic fatty acid precursors into a range of LC-PUFA up to and including DHA, and initial results of LC-PUFA production in plants have been published (6,7). A major challenge associated with this approach is the channeling of the products of these individual enzymes to the next desired reaction (8).

The cultivation of microbial organisms has provided an alternative commercial source of oils enriched in LC-PUFA. The fungus *Mortierella alpina* and a dinoflagellate, *Crypthecodinium cohnii*, are both currently grown via fermentation as sources of oils enriched in LC-PUFA [arachidonic acid, ARA (C20:4 ω-6), and DHA, respectively] that are added to infant formulas (9, 10). The microalga *Schizochytrium* sp., as well as other strains of Thraustochytrids, can produce large amounts of oil—up to 55% of the cell weight—in which DHA can comprise as much as 35% of the total fatty acids (11). *Schizochytrium* sp. is grown on a commercial scale via fermentation for both biomass (for animal feed) and for its oil (11).

Until recently, it was assumed that all LC-PUFA were produced by variations of the same basic pathway mentioned above. That is, an FAS system produces short chain saturated fatty acids, and these products are subsequently modified by the actions of distinct elongase and desaturase enzymes. Indi-

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Abbreviations: ACP, acyl carrier protein; ARA, arachidonic acid (C20:4 ω-6); bp, nucleotide base pair; DHA, docosahexaenoic acid (C22:6 ω-3); DPA, docosapentaenoic acid (C22:5, this study refers exclusively to DPA ω-6); EPA, eicosapentaenoic acid (C20:5 ω-3); IPTG, isopropyl-1-thio-β-D-galactopyranoside; KAS, β-ketoacyl-ACP synthase; LC-PUFA, those PUFA with carbon chain lengths greater than 18; Orf; open reading frame; Orf B*, *Schizochytrium* PUFA synthase Orf B modified for expression in *E. coli*; PKS, polyketide synthase; PPTase, phosphopantetheinyl transferase; RBS, ribosome binding site.

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cations of the existence of an alternative pathway for LC-PUFA synthesis initially came from studies of certain marine bacteria. In 1986, DeLong and Yayanos (12) reported the detection of LC-PUFA (either EPA or DHA) in several strains of psychrophilic marine bacteria. Subsequently, Yazawa (13) identified a segment of genomic DNA from an EPA-producing bacterium (*Shewanella* strain SCRC-2738) that when transformed into *E. coli* resulted in EPA accumulation in those cells. It was then determined that proteins encoded by five open reading frames (Orfs) were necessary and sufficient for this activity (14). A number of observations led to the conclusion that four of those proteins represented subunits of an enzyme complex capable of *de novo* synthesis of EPA (1). This PUFA synthase enzyme possessed multiple domains, some of which showed homology to those found in FAS systems while others were similar to those of polyketide synthase (PKS) systems. The fifth essential gene required for EPA synthesis in *E. coli* encoded a phosphopantetheinyl transferase (15) that activated the acyl carrier protein (ACP) domains present on the enzyme by attachment of a cofactor (16). Genes with homology similar to the *Shewanella* EPA gene cluster have been identified in other marine bacteria, including one which accumulates DHA (16-18). It is possible that most, or all, of the marine bacteria that synthesize LC-PUFA will be found to utilize this system.

A set of three genes encoding a PUFA synthase homologous to those found in marine bacteria was also discovered in *Schizochytrium*. A number of biochemical and physiological observations, in addition to the molecular data, indicated that the abundant LC-PUFAs that accumulate in this organism are the products of a PUFA synthase, or synthases (1, 11). Here we confirm that the three genes identified in *Schizochytrium* do indeed encode subunits of a PUFA synthase and that this synthase accounts for production of both DHA and DPA. Additionally, we provide evidence that the other abundant fatty acids present in *Schizochytrium* oil (C14:0 and C16:0) are the products of a separate FAS. With regard to both sequence homology and domain organization, the *Schizochytrium* FAS resembles those found in fungi. However, in contrast to the two subunits of the fungal FAS, all of the domains of the *Schizochytrium* FAS are found on a single large protein.

EXPERIMENTAL PROCEDURES

Strains. *Schizochytrium* sp. is available from the American Type Culture Collection (ATCC 20888) as are *Nostoc* sp. PCC7120 (ATCC 27893, deposited as *Anabaena* sp.) and *Bacillus subtilus* (ATCC 21332). The *E. coli* strain BL21 [BL21(DE3)] was obtained from Novagen.

General preparation of E. coli transformants. DNA containing Orfs encoding the *Schizochytrium* PUFA synthase subunits A, B, and C were cloned separately and together into *E. coli* expression vectors derived from pET21c (Novagen). The constructs were transformed into BL21 cells and expressed using the T7 system with IPTG (isopropyl-1-thio-β-D-galactopyranoside) induction (Novagen). Initial assembly of the Orfs, each containing modifications to facilitate cloning, was done in the pBluescript II SK(+) plasmid (Stratagene, Inc.). The fragments used to assemble the PUFA synthase subunits, as well as the *Schizochytrium* FAS, were cloned from a lambda library of genomic DNA. Standard methods were used to produce that library as well as polymerase chain reaction (PCR)-derived probes (based on sequences obtained from cDNA clones) for isolation of the respective DNA fragments. Sequences for the Orfs encoding PUFA synthase subunits are available from GenBank (accession numbers: AF378327, AF378328, AF378329 for subunits A, B, and C, respectively). The GenBank sequences provide the reference base pair (bp) positions and restriction site locations described in the cloning steps listed below. The Orf encoding the FAS gene was sequenced using a combination of subcloning and primer walking. The accession number for the *Schizochytrium* FAS Orf is EF015632.

Orf A was cloned as three pieces. Part 1 included the beginning of the Orf and extended to a unique PstI site 18 bp from the start codon. This fragment was generated using two complimentary oligonucleotides that were designed to add EcoRI and PacI restriction enzyme sites and a bacterial ribosome binding site (RBS) upstream of the start codon. Additionally, they incorporated an NdeI site at the start codon. Part 2 was a 1.2-kb fragment extending from the PstI site to a unique XmaI site at 1243 bp. Part 3 was a 7.5-kb fragment extending from the XmaI site to a SpeI site 6 bp downstream of the stop codon. Parts 2 and 3 were assembled into pBluescript II cut with PstI and SpeI. Part 1 was added to this construct as an EcoRI-PstI fragment generating the modified Orf A clone.

Orf B was cloned in four parts. Part 1 was generated by PCR and included the beginning of the Orf, and extended to a BsiWI site 237 bp from the start of the Orf. The 5′ primer included an EcoRI site followed by an RBS and an NdeI site at the start codon. The 3′ primer included a SacII site to facilitate further cloning. The PCR product was digested with EcoRI and SacII and cloned into pBluescript II digested with the same restriction enzymes. Part 2 was a 4.36-kb restriction fragment extending from the BsiWI site to a SacII site 4605 bp into the Orf. Part 3 was a gel-purified 1.54-kb restriction fragment extending from the SacII site at 4605 bp to a ClaI site 6147 bp into the Orf. Part 4 extended from the ClaI site at 6147 bp to the end of the Orf. This short (68 bp) fragment was generated using two overlapping oligonucleotides that included EcoRI and SacII sites upstream of the ClaI site and a BamHI site just downstream of the stop codon to facilitate further cloning. The modified Orf B was assembled in the following sequence. The cloned part 1 was digested with BsiWI and SacII and ligated together with the gel-purified part 2 fragment. The new clone was digested with EcoRI and SacII, and the 4.6-kb fragment gel purified. The part 4 clone was digested with SacII and ClaI, and ligated together with the gel-purified part 3 fragment. This plasmid was opened up by digestion with EcoRI and SacII and ligated together with the 4.6-kb gel-purified fragment containing parts 1 and 2.

Orf C was cloned in four parts. Part 1 contained the start codon and extended to a NarI site 255 bp into the Orf. This fragment was generated by PCR. The 5′ primer included an

EcoRI site followed by an RBC and an NdeI site at the start codon. The primer for the 3′ end contained a BamHI site just beyond the NarI site to facilitate further cloning. The PCR product was digested with EcoRI and BamHI and ligated into pBluescript II digested with the same restriction enzymes. Part 2 was a 0.7-kb restriction fragment extending from the NarI site to a BamHI site 971 bp into the Orf. Part 3 was a 3.3-kb restriction fragment extending from the BamHI site to a PflMI site at 4248 bp. Part 4 extended from the PflMI site to the end of the Orf. This fragment was generated by PCR using primers that added BamHI and EcoRI sites upstream of the PflMI site and NheI, HindIII, and XbaI sites downstream of the stop codon to facilitate further cloning. The PCR product was digested with BamHI and XbaI and ligated into pBluescript digested with the same enzymes. The modified Orf C was assembled using the following steps. The plasmid containing part 1 was digested with NarI and BamHI and ligated together with the gel-purified part 2 fragment. This plasmid was digested with EcoRI and BamHI, and the 1-kb fragment containing parts 1 and 2 was gel purified. The plasmid containing part 4 was digested with PflMI and BamHI and ligated together with the part 3 fragment. The plasmid containing parts 3 and 4 was opened up by digestion with EcoRI and BamHI and ligated together with the fragment containing parts 1 and 2.

Cloning of modified Orfs A, B, and C individually into a modified pET21c and testing for protein expression in E. coli. To facilitate cloning into pET21c, restriction sites for NheI, PacI, PmeI, and SpeI were added, in that order, to the multiple cloning region between the XhoI and BlpI sites using two adapter oligonucleotides. The modified vector (pMET21) was used for the cloning of the individual, as well as multiple, PUFA synthase Orfs. The modified PUFA synthase Orf A was cut out of pBluescript II with NdeI and SpeI. The resulting 8.7-kb fragment was gel purified and ligated into pMET21 cut with the same enzymes. The modified Orf B was cut out of pBluescript II with NdeI and BamHI. The resulting 6.2-kb fragment was gel purified and ligated into pMET21 cut with NdeI and BamHI. The modified Orf C was cut out of pBluescript II with NdeI and HindIII. The resulting 4.5-kb fragment was gel purified and ligated into pMET21 cut with NdeI and HindIII.

BL21 cells carrying the various pMET21 constructs were grown in Luria Broth plus ampicillin (100 μg/mL) at 32°C with shaking to an A_{600} of 0.5. IPTG was added to a final concentration of 1 mM, the cells grown for an additional four hours, and then harvested by centrifugation. The cell pellets were heated in an SDS sample buffer and the extracted proteins analyzed by SDS-PAGE (sample buffer, 4-20% polyacrylamide Tris-glycine gels, and Simple Blue stain, all from Novex).

Resynthesis of a serine codon TCT repeat region in Orf B and testing for protein expression. Orf B contains a stretch of 15 consecutive, identical serine codons (TCT), located at 4510 to 4554 bp. An approximately 200-bp segment, containing the region from a BspHI site at 4416 bp to the SacII site at 4605 bp, was resynthesized by use of PCR and a series of partially overlapping oligonucleotides, using the two-stage amplification protocol outlined in reference 19 as a guide. The final PCR product was cloned and sequenced and the BspH-SacII fragment gel purified for subsequent cloning. This fragment replaced the native region of Orf B (forming Orf B*) using the steps outlined for the general preparation of the Orf B clone described above. The pBluescript clone containing part 1 was cut with BsiWI and SacII. Into this was ligated a 4.2-kb BsiWI-BspHI fragment derived from the clone containing parts 1 and 2, plus the resynthesized BspHI-SacII fragment. Digestion of this clone with EcoRI and SacII yielded a 4.6-kb fragment which was gel purified and ligated into the clone of parts 3 and 4 digested with the same enzymes. This clone contained the complete, modified Orf B*, which was then transferred to pMET21, using the same cloning strategy as described above, and tested for protein expression.

Cloning of Orfs A, B, and C into pMET21, and construction of pMET21-Orf B/Orf C/Orf A.* Starting with the pMET21-Orf B clone, Orfs C and A were sequentially added downstream of the previously inserted genes. This created a polycistronic construct with the expression of all three genes driven by the single T7 promoter in pMET21. Orf B utilized the vector-encoded RBS, while a new RBS was supplied with Orfs C and A. A 4.5-kb EcoRI-NheI fragment isolated from pMet21-Orf C (containing Orf C plus an upstream RBS), was ligated into pMET21-Orf B cut with the same enzymes. The resulting pMET21-OrfB/Orf C was digested with PacI and SpeI. Into this was ligated an 8.7-kb PacI/SpeI fragment containing Orf A plus an upstream RBS, isolated from pMet21-Orf A. The resulting plasmid, pMET21-Orf B/Orf C/Orf A was used as the source of a 13.2-kb EcoRI-SpeI Orf C/Orf A fragment used to construct pMET21-Orf B*/Orf C/Orf A. This construct was generated by digesting pMET21-Orf B* with EcoRI and SpeI and ligating in the gel-purified fragment.

Cloning of sfp and HetI into pACYC184. Two PPTases were tested in the *E. coli* PUFA synthase expression system: sfp, from *Bacillus subtilis* (20), and HetI, from the cyanobacterium *Nostoc* strain 7120 (21). An expression vector for sfp was built by cloning the coding region along with defined upstream- and downstream-flanking DNA into pACYC184 (New England Biolabs, Inc.). The oligonucleotides: CGGGGTACCCGGGAGCCGCCTTGGCTTTGT (forward); and AAACTGCAGCCCGGGTCCAGCTGGCAGGCACCCTG (reverse), were used to amplify this region from genomic *B. subtilus* DNA. Restriction enzyme sites were included in the oligonucleotides to facilitate cloning into the EcoRV site of pACYC184. This pACYC184-sfp clone was used to create a HetI expression cassette. The sfp coding region (along with three nucleotides immediately upstream of the ATG) was replaced with a section of DNA containing NdeI and XhoI restriction sites. The ATG sequence embedded in the NdeI site was utilized as the initiation methionine codon for the introduced HetI Orf. There are no methionines present in the Orf, but there are several potential alternative start codons (TTG and ATT) near the 5′ end (see reference 21, Figs. 2 and 3). PCR was used to amplify the Orf with primers designed to replace the furthest 5′ TTG codon with an ATG codon as part of the above-mentioned NdeI site. PCR was also used to introduce an XhoI site just beyond the stop codon. The modified HetI

coding sequence was then inserted into the NdeI and XhoI sites of the pACYC184 vector construct containing the sfp regulatory elements. Upon introduction into BL21 cells, transformants were selected based on chloramphenicol (35 μg/mL) resistance.

Fatty acid content of E. coli cells expressing Schizochytrium PUFA synthase subunits A, B, and C plus sfp or HetI. BL21 cells containing pMET-OrfB*/OrfC/OrfA and either pACYC184-sfp or pACYC184-HetI were grown in various media supplemented with ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL) to A_{600} = 0.5. IPTG was then added to a final concentration of 0.5 mM. Incubation was continued for 24 hours and the cells were harvested by centrifugation, washed in 50 mM Tris pH 7.5, and processed for FAME analysis. The following media were tested: Luria Broth, Luria Broth supplemented with 5- or 10% glycerol and 765 medium (recipe available from the ATCC) supplemented with 10% (wt/vol) glycerol. Cells were grown at 20°C or 32°C with shaking. Cells transformed with pMET-OrfC/OrfA served as the negative control.

Fatty acids present in the culture were converted to FAMEs and identified by comparison of retention times of standards using GC-FID. FAMEs were prepared by adding 2 mL of 1.5 N anhydrous HCl in methanol and 1 mL toluene to aliquots of the wet pellets and heating at 100°C for two hours with frequent mixing. After cooling to room temperature, 1 mL of NaCl-saturated water was added, the samples mixed, and the organic layer removed for analysis. Determination of carbon-carbon double bond positions was made by interpretation of MS data of dimethyloxazoline (DMOX) derivatives of the FAME samples (22). 100 μL of the FAME solution was transferred to screw top test tubes and the solvent removed with nitrogen gas. One mL of 2-amino-2-methyl propanol was added, the tubes flushed with nitrogen, capped, and heated at 150°C for 18 hours. The residue was dissolved in 5 mL of dichloromethane, 5 mL of water was added, and the aqueous layers were removed after thorough mixing. The wash step was carried out five times. The dichloromethane solution was dried over anhydrous sodium sulfate, filtered, and concentrated using nitrogen gas to 1 mL final volume. Identification of fatty acids in the DMOX derivatives was based on comparison with published data when available, and with the interpretation of MS of individual sample peaks.

Effects of cerulenin on ^{14}C acetate labeling of fatty acids in Schizochytrium cells. Schizochytrium cells were grown in a medium designed to promote oil accumulation (23), with shaking at 27ºC, until the start of logarithmic growth (A_{600} of ~3.0). Cerulenin (Sigma) dissolved in EtOH was added to glass tubes and the solvent evaporated prior to adding 1 mL aliquots of the culture. The amount of cerulenin in the tubes represented final concentrations of 0, 0.5, 1.0, 5.0, 10, 25, 50,100, or 200 μM in the cell culture. The cells were incubated for 10 minutes prior to the addition of ^{14}C acetate (39 mCi/mmol: Sigma) to a final concentration of 20 μM. Incubations were continued until >90% of the label had been removed from the medium (~45 minutes). Cells were collected by centrifugation, and FAMES were prepared essentially as described above. The solvent was removed with nitrogen gas and the residue dissolved in hexane and applied to silica gel G TLC plates (J.B. Baker) that had been treated with a 10% silver nitrate solution. Plates were developed with hexane/diethyl ether/acetic acid (70:20:2). Radioactive areas on the plates were detected using a phosphorimaging system (BioRad, FX Pro Plus). The intensities of photon emissions from the storage phosphor screens were used to measure the relative amounts of radioactive material present and are expressed as "counts" in rectangles of equivalent areas. Identities of the FAMEs on the plates were established by comparison to unlabeled FAME standards (C14:0, C16:0, DPA, and DHA: NuCheck Prep). The C14:0 and C16:0 standards co-migrated on the plates near the solvent front, while DPA and DHA ran as separate bands near the origin.

RESULTS

Expression of Schizochytrium PUFA synthase subunits A, B, and C in E. coli. We sought to confirm that the *Schizochytrium* Orfs A, B, and C encode a PUFA synthase and to verify its fatty acid products by expressing that system in *E. coli*. The PUFA synthase genes previously expressed in *E. coli* or in a cyanobacterium are from marine bacteria, and the Orfs encoding the subunits of those PUFA synthases are organized in apparent operons. Expression of those systems in *E. coli* utilized the promoter, initiation, and translation elements of the source DNA (13, 14, 16). The eukaryotic nature of the *Schizochytrium* genes precluded this approach. We utilized the Novagen pET vectors to express the genes encoding subunits A, B, and C individually and together as a synthetic operon. SDS-PAGE analyses of extracts from *E. coli* in which the individual genes were induced revealed that the genes encoding subunits A and C produced proteins of the expected sizes. In contrast, extracts from cells expressing the subunit B gene accumulated a novel protein with an apparent mass of 165 kDa instead of the 224 kDa protein predicted by translation of the coding sequence (data not shown). Examination of the Orf B nucleotide sequence revealed a region containing 15 sequential identical serine codons (TCT) at a position in the Orf that could account for generation of the truncated protein.

A 200-bp fragment of Orf B containing the serine repeat region was resynthesized so that a random mix of the three serine codons commonly used by *E. coli* replaced the TCT repeat. Some other potentially problematic codons in this fragment were also changed while maintaining the original deduced amino acid sequence (Fig. 1). The native fragment of Orf B was replaced with the modified one (creating Orf B*) and expressed in *E. coli*. In this case an apparently full-length subunit B protein was detected. Analysis of cells expressing the construct containing all three Orfs (A, B*, and C) revealed the presence of three new proteins of the expected sizes. Analysis of the fatty acids in these cells did not reveal any LC-PUFA.

Selection and cloning of a PPTase-HetI. Subunit A of the *Schizochytrium* PUFA synthase contains nine adjacent, highly homologous, ACP domains. ACPs require conversion from an inactive apo-form to a functional holo-form by attachment of a

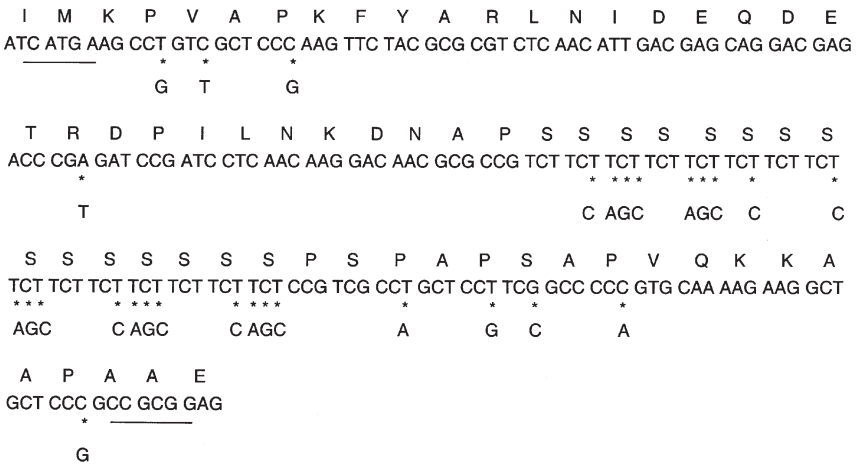

FIG. 1. Modification of the *Schizochytrium* PUFA synthase Orf B nucleotide sequence for expression in *E. coli*. The sequence of codons associated with a BspHI-SacII fragment (restriction enzyme recognition sites are underlined) is shown, along with the encoded amino acids. Nucleotides changed in the resynthesized fragment are indicated by *, with the altered nucleotide shown below. The first and last nucleotides shown correspond to positions 4414 and 4608, respectively, of the complete *Schizochytrium* Orf B sequence (Accession number, A378328).

phosphopantetheine prosthetic group. Enzymes of the PPTase superfamily catalyze this post-translational modification (15). Previous work suggested that *E. coli's* endogenous PPTases would be incapable of activation of the ACPs of PUFA synthases (16). A PPTase that was likely to meet our requirements was identified by searching for ones that may activate ACP domains with significant homology to those of the PUFA synthases. The *hetI* gene is presumed to encode a PPTase (HetI) and is found in a cluster of genes in *Nostoc* sp. responsible for the synthesis of long-chain hydroxy-fatty acids (21, 24). It is likely that HetI activates the two ACP domains of the protein encoded by the *hglE* gene present in that same cluster. The ACP domains of HglE have a high degree of sequence homology to each other and to the ACP domains found in *Schizochytrium* Orf A as well as those of other PUFA synthases (Fig. 2). The amino acids in the region immediately surrounding the phosphopantetheine attachment site are identical in these ACP domains, and there is significant homology in the extended sequence. In contrast, the *E. coli* ACP has relatively few residues that are identical to those in this alignment. To our knowledge, HetI has not been tested previously in a heterologous system, and the endogenous start codon of HetI has not been identified. There are no ATG codons in the potential coding region, but there are several alternative bacterial start codons (TTG and ATT) near the 5′ end of the Orf (21). A HetI expression construct was made by replacing the farthest 5′ alternative start codon (TTG) of the Orf with a methionine codon. Regulatory elements obtained from the upstream region of the *sfp* gene of *B. subtilus* were used to drive expression of the gene. SDS-PAGE analysis of extracts from *E. coli* transformed with this plasmid revealed the appearance of a new protein of the appropriate size (not shown). The gene-encoding sfp has been used extensively for activation of ACP domains of PKS systems as well as the peptidyl carrier protein domains of non-ribosomal peptide synthase systems during heterologous expression (15). We also tested the effect of expression of sfp with the *Schizochytrium* PUFA synthase genes in *E. coli*.

Accumulation of DHA and DPA in E. coli expressing the Schizochytrium Subunits A, B, and C plus HetI or sfp. E. coli cells were transformed with various plasmids containing some or all of the putative *Schizochytrium* PUFA synthase genes and with a second plasmid containing a PPTase gene. Transcription of the genes dependent on the T7 polymerase was induced by addition of IPTG, the cultures incubated overnight, and the fatty acids analyzed. A typical GC profile of FAMEs derived from control cells (lacking at least one component of the PUFA synthase system) is shown in Fig. 3A. The major peaks on the chromatogram were identified as 16:0, 16:1, 18:0, and 18:1 (*cis*-vaccenic acid), as well as two cylcopropane fatty acids (17:0 and 19:0). No peaks corresponding to DHA or DPA, or any other PUFA, were detected in these samples. In contrast,

FIG. 2. Alignment of the highly conserved central regions of the first ACP domains from a heterocyst glycolipid synthase of *Nostoc* sp. PCC7120 (HglE), the PUFA synthase from *Shewanella* SCRC3728 (Orf 5), and the PUFA synthase of *Schizochytrium* (Orf A). Identical amino-acid residues are indicated by the grey background. Underlined amino acids indicate residues of the *E. coli* ACP that are identical to those in this alignment. An * marks the conserved serine pantetheinylation site. The accession numbers and the range of amino acid sequences used are the following: HglE, BAB77050, 1320–1353; Orf 5, AAB81123, 1269–1302; Orf A, AAK72879, 1135–1168; *E. coli*, AAC74178, 15–48.

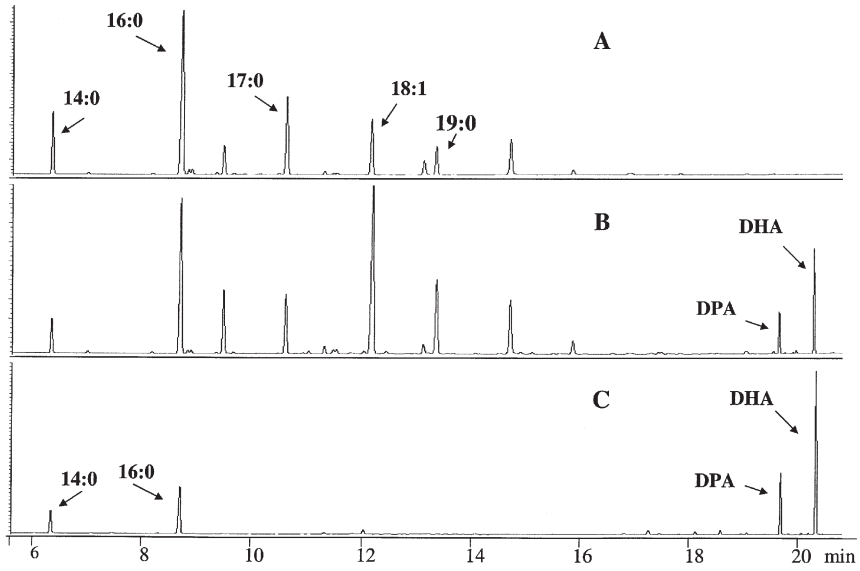

FIG. 3. GC profiles of FAMEs prepared from the following: A) control *E. coli*; B) *E. coli* expressing Orfs A, B, and C of the *Schizochytrium* PUFA synthase plus HetI; and C) oil extracted from *Schizochytrium*. The horizontal axis represents retention time on the column, and the vertical axis the relative response signal of the detector. Identities of the labeled FAME peaks have been confirmed by GC-MS. The 18:1 peak is *cis*-vaccenic acid. The *Schizochytrium* oil FAME profile is shown for comparison to the novel fatty acids produced in the *E. coli* expressing the *Schizochytrium* PUFA synthase system.

expression of *Schizochytrium's* Orfs A, B, and C, along with either HetI or sfp—under several conditions—resulted in the accumulation of DHA and DPA in those cells. The highest level of PUFA (DHA plus DPA), often representing greater than 10% by weight of the total FAME, was found in cells with HetI grown at 32°C in 765 medium supplemented with 10% (wt/vol) glycerol (Fig. 3B). In all of the experiments in which the two PPTases, sfp and HetI, were compared, significantly more DHA and DPA accumulated in the cells containing HetI than in cells containing sfp. SDS-PAGE analysis suggested approximately equivalent amounts of the proteins were being produced, but we did not attempt further analysis of the relative enzyme activities. Fig. 3C shows a typical GC separation of FAMEs prepared from purified *Schizochytrium* oil. There are four prominent peaks in the chromatogram, representing C14:0, C16:0, DPA, and DHA. The ratio of DHA to DPA observed in *E. coli* expressing this PUFA synthase system is about 2.3 to 1. This approximates that of the endogenous DHA and DPA production observed in *Schizochytrium* itself.

Cerulenin differentially affects fatty acid synthesis in Schizochytrium. Rapidly growing cells were treated with various amounts of the β-ketoacyl-ACP synthase inhibitor, cerulenin, for ten minutes prior to the addition of and incubation with ^{14}C-labeled acetate. Radiolabeled fatty acids present in those cells were converted to FAMEs, separated by TLC, and quantitated using a phosphorimaging system. Three major bands, identified as DHA, DPA, and short-chain-saturated (C16:0 and C14:0) FAMEs, were evident in the image, and all were affected by the cerulenin at some concentration. The inhibitor affected incorporation of labeled acetate into DHA and DPA equally and required high concentrations (>5 μM), while labeling of the short-chain-saturated fatty acids was blocked at lower concentrations (Fig. 4). In some cases, for example, at 5, 10, 25, and 50 μM, there is significant labeling of the PUFAs, while labeling of the short-chain saturates has been severely inhibited. The results indicate a separate pathway for synthesis

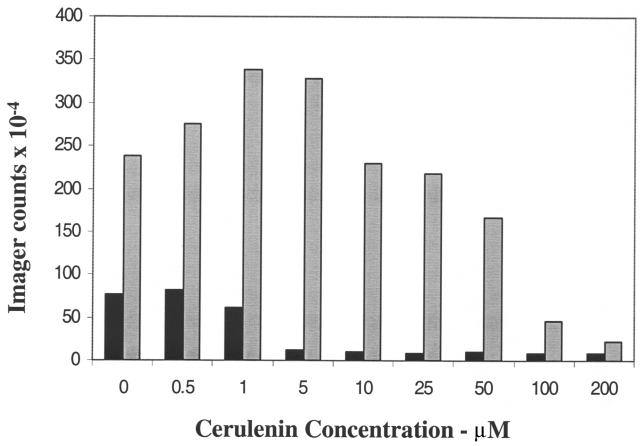

FIG. 4. Relative incorporation of radioactivity into PUFAs (DHA plus DPA - grey bars) or short-chain saturated fatty acids (C14:0 plus C16:0 - black bars) from ^{14}C acetate by *Schizochytrium* cells preincubated with various concentrations of the FAS inhibitor, cerulenin. Imager counts are values in equal-sized areas of the phosphorimager data collected from a scan of a TLC plate on which FAMEs derived from the cells were separated.

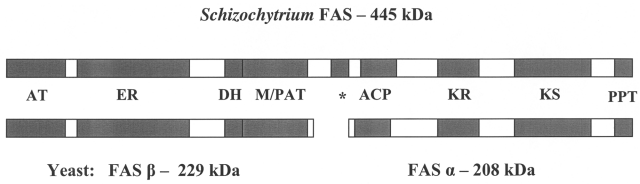

FIG. 5. The apparent domain organization of the *Schizochytrium* FAS (all on a single protein) with that of *Saccharomyces cerevisiae* FAS (domains distributed on the β and α subunits). Relative sizes of the domains reflect the results of Pfam profiling. Domain indicators refer to both *Schizochytrium* and yeast FAS proteins. The * indicates the location in the *Schizochytrium* FAS of a putative ACP domain not present in the yeast FAS. The following are abbreviations used: AT, acetyltransacylase; ER enoyl-reductase; DH, dehydratase; M/PAT, malonyl/palmitoyl transacylase; ACP, acyl carrier protein; KR, β-ketoacyl reductase; KS, β-ketoacyl synthase; PPT, phosphopantetheinyl transferase.

of the two major types of fatty acids that accumulate in *Schizochytrium*.

Cloning of the gene encoding the Schizochytrium FAS. Homologs of bacterial PUFA synthase genes were initially detected in *Schizochytrium* by analysis of sequences obtained from randomly picked cDNA library clones (1). Further analysis of the cDNA library identified many sequences (43 out of approximately 8,500) that showed homology to proteins of FAS systems. The best matches were, in most cases, to the FAS subunits of fungal organisms. Using the sequence tags as a guide, we cloned the corresponding regions of genomic DNA encoding those cDNA-derived sequences. A single large Orf was found in the genome that contained all of the EST sequences. The *Schizochytrium* FAS Orf contains 12,408 bp and encodes a protein with a deduced molecular mass of 444,884 Daltons. No evidence for introns could be found, either by analysis of the genomic sequence itself or by comparison to available cDNA sequences. Blast and Pfam results plus motif analyses were used to establish a preliminary structure and functional identification of the domains of this Type I protein. Both in terms of amino acid sequence and in the sequential organization, the *Schizochytrium* FAS resembles a fusion of the head (N-terminus) of the fungal FAS α subunit to the tail (C-terminus) of the β subunit (Fig. 5, the well-characterized *Saccharomyces cerevisiae* FAS shown for comparison). This similarity includes regions corresponding to an acetyltransferase initiation domain, a malonyl/palmitoyl acyltransferase domain, and a terminal PPTase domain. One other difference is the presence of what may be a second ACP domain at the fusion junction.

DISCUSSION

Schizochytrium sp. is a member of the Thraustochytrids, a group of marine microorganisms taxonomically related to the heterokont algae (25). Organisms of this group have attracted attention recently due to their ability to produce large amounts of oil enriched in DHA (11). Additionally, several genes encoding key enzymes of the standard pathway of PUFA synthesis, including a Δ-4 desaturase, have been cloned from the Thraustochytrid species closely related to *Schizochytrium* (26). It was somewhat surprising, therefore, that homologs of PUFA synthase genes, previously identified only in marine bacteria were found in *Schizochytrium*. Although there are some differences between the bacterial systems and the one found in *Schizochytrium*, all of the domains known to be required for PUFA synthesis in bacteria have been identified (1). One scenario that could account for the appearance of these genes in this eukaryote is a lateral gene transfer from the bacteria. Thraustochytrids are known to be phagocytotic; bacteria can be used as a food source, and this may have facilitated such a process. The isolation of genes encoding enzymes involved in the synthesis of LC-PUFA from shorter-chain, less unsaturated fatty acid precursors suggests that Thraustochytrids are, or were, capable of DHA synthesis via that pathway. When cultures of *Schizochytrium* sp. were supplied with radiolabeled 16:0, 18:1, or 18:3 fatty acids, none of the label appeared in LC-PUFA (1), indicating that at least some elements of the standard pathway have been lost in this particular organism. As more Thraustochytrids are studied, research may reveal a range of expression of these distinct LC-PUFA synthesis pathways.

The PUFA synthases characterized in marine bacteria appear to produce predominantly a single product: either EPA or DHA. In contrast, the *Schizochytrium* fatty acid profile contains significant amounts of two LC-PUFA: DHA and DPA (Fig. 3C). Based on the lack of evidence for more than one set of PUFA synthase genes, we predicted that both fatty acids would be the product of one enzyme system. This prediction was confirmed by the accumulation of both DHA and DPA in *E. coli* expressing the *Schizochytrium* genes (Fig. 3B).

The PPTases present in *E. coli* are apparently unable to modify the apo-ACP domains of the PUFA synthases. It is now recognized that the detection by Yazawa of EPA in *E. coli* transformed with a genomic fragment of *Shewanella* SCR2738 DNA was made possible because the gene encoding that PUFA-synthase-specific PPTase was also present on the fragment (15, 16). We have not identified a gene in *Schizochytrium* that encodes a PUFA-synthase-specific PPTase. While the *Shewanella* PPTase has been shown to activate other PUFA synthase enzymes (16), the psychrophilic nature of that organism suggested it might not be appropriate for our purposes. The nitrogen-fixing cyanobacterium, *Nostoc* PCC7120 (formerly called *Anabaena*), grows well at relatively high temperatures (e.g., 30ºC) and contains a cluster of genes associated with the synthesis of a long-chain hydroxyl-fatty acid that forms part of an oxygen-excluding glycolipid layer of the heterocysts (21). Many of the domains present in the proteins encoded by these genes, including 2 ACP domains, are highly homologous to those of PUFA synthases. The *hetI* gene, encoding a presumed PPTase, is adjacent to this fatty acid synthesis cluster. As in the case of the *Shewanella* PPTase, it is not clear from the nucleotide sequence what the endogenous start codon is for *hetI*. We found that an active enzyme could be produced by replacing the first potential alternative (bacterial) start codon in the HetI Orf with an ATG in an expression construct. HetI, like sfp, may have utility in activating ACP domains of a variety of PKS

systems in addition to those of the PUFA synthases when they are expressed in heterologous hosts.

While it is clear that the LC-PUFAs in *Schizochytrium* are the products of a PUFA synthase, little is known about the source of the short-chain saturated fatty acids (C16:0 and C14:0) that are also prominent components of the TAG fraction. To our knowledge, no characterization of FAS systems in Thraustochytrids has been published by others. Cerulenin is a fungal antibiotic that irreversibly inhibits β-ketoacyl-ACP synthases (KAS) of FAS systems (27). The sensitivity of specific KAS enzymes to this antibiotic varies widely. Cerulenin has been used to enhance PUFA accumulation in marine bacteria that contain PUFA synthase systems (28), presumably by selectively inhibiting the Type II FAS in those bacteria. The differential effect of cerulenin on incorporation of ^{14}C-acetate into short-chain fatty acids versus PUFA in whole cells of *Schizochytrium* (Fig. 4) confirmed our expectation that these fatty acids are products of separate pathways. EST data suggested the presence of a Type I FAS in *Schizochytrium*. In all cases, the best matches to the EST sequences were to FAS proteins found in fungi. In these organisms the Type I FAS is composed of two subunits (α and β), each carrying a distinct set of enzymatic domains (29). In contrast, we found that the *Schizochytrium* FAS contains all of these domains on one large protein. Animal FASs contain all of their catalytic domains on one large protein, but the domain arrangement and both the loading and termination mechanisms differ from that found in the fungal FAS (29). As was the case for the PUFA synthase, the EST data indicate that there is a single copy of the FAS gene in *Schizochytrium* and that messages derived from that gene are highly expressed in oil-producing cells.

In summary, our data confirm that the DHA and DPA present in the oil of *Schizochytrium* are the products of a single PUFA synthase enzyme. It is likely, though yet to be confirmed by detailed molecular analyses, that the genes encoding this enzyme were acquired at some time in the past from an LC–PUFA-producing marine bacterium. The data suggest that the two short-chain saturated fatty acids (C14:0 and C16:0) that are the other major components of the *Schizochytrium* oil, are the products of an FAS enzyme encoded by the gene we have cloned and characterized. The FAS encoded by this gene shows strong resemblance to many other fungal FAS proteins, but has the unique feature of having all of its domains present on one large protein.

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Different Kinetic in Incorporation and Depletion of n-3 Fatty Acids in Erythrocytes and Leukocytes of Mice

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ABSTRACT: n-3 PUFA are well known for their anti-inflammatory effects. However, there has been only limited study on the kinetics of incorporation and depletion of n-3 PUFA in immune cells. In the present study we investigated the incorporation and depletion of n-3 PUFA in erythrocytes and leukocytes in mice during a 6-wk feeding period. Over the first 3-wk period (the incorporation period) the mice were fed a special diet with a high n-3/n-6 PUFA ratio. In the following 3-wk period (the depletion period) the mice were fed a standard chow diet. A linear increase of the concentration of EPA and DHA in erythrocyte membranes was observed during the incorporation period, whereas a stagnation was observed after the second week for leukocytes. The level of EPA did not fall to the background level after the depletion period, and the level of DHA was kept almost constant during the depletion period in the erythrocyte membranes. In leukocytes the concentration of both EPA and DHA decreased during the depletion period, but did not reach the background level after the 3-wk depletion. In conclusion, the kinetics of EPA and DHA in the different cells are different. The rate of incorporation is faster than that of depletion for n-3 PUFA. More n-3 PUFA can be incorporated into leukocytes in comparison with erythrocytes. The ratio of n-3/n-6 PUFA is more important than the amount of n-3 FA in changing the FA compositions of membrane lipids.

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Fatty fish contain high concentrations of long-chain n-3 PUFA such as EPA (20:5n-3) and DHA (22:6n-3). A high intake of these FA has been correlated with beneficial healthy effects such as reduced risk of coronary heart disease (1,2) and decreased inflammation in rheumatoid arthritis (3). The concentration of both n-3 PUFA (DHA and EPA) and the n-6 PUFA arachidonic acid (AA, 20:4n-6) and linoleic acid (LA,18:2n-6) of erythrocyte phospholipids have been suggested to be the best markers of n-3 PUFA intake (4).

Animal feeding experiments have been widely used in biochemical and nutritional studies of n-3 PUFA. Even though there are many reports about tissue FA changes in response to dietary enrichment with n-3 PUFA (2,5,6), only limited information about the kinetics of incorporation and depletion of n-3 PUFA in immune cells existed in the literature (7,8). In the present study, we investigated the kinetics for incorporation and depletion of n-3 PUFA in both erythrocytes and leukocytes in mice. The results of this study contribute to a better understanding of lipid metabolism, and may also have potential implications for clinical nutrition.

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Abbreviations: AA, arachidonic acid; LA, linoleic acid.

MATERIALS AND METHODS

Animals, diets and experimental design. The experiment was approved by The Danish National Committee for Animal Experiments and conducted with 8-wk-old BALB/c female mice (Taconic M&B, Lyngby, Denmark). The mice were housed in polyethylene cages in a temperature (21 ± 3ºC) and humidity (50 ± 15%) controlled room with a 12-h day-night rhythm at arrival. Thirty-six mice were randomly assigned into nine experimental groups with four mice in each group: three control groups on standard chow (Altromin 1324, Chr. Petersen A/S, Ringsted, Denmark), and six groups on special diet. The overall composition of the special diet was as follows (wt%): 56% maize-starch (Bestfoods Nordic A/S, Skovlunde, Denmark), 4% fat, 20% casein (Miprodan milkproteins, MD Foods, Viby, Denmark), 10% sucrose (Danisco Sugar, Copenhagen, Denmark), 5% salt mixture (including trace elements), 4% cellulose powder (MN 100, Frisenette ApS, Ebeltoft, Denmark), 0.5% choline chloride (Merck, Darmstadt, Germany), and 0.5% vitamin mixture (Aaes-Jørgensen and Hølmer). The control diet was Altromin (1324, Chr. Petersen, Ringsted, Denmark), which also contained 4% fat. The FA composition of each of the diets is listed in Table 1. The ratio of n-3/n-6 PUFA was 2.4 for the special feed, in which EPA and DHA constituted 2.2% and 3.5% of total FA, respectively, whereas the ratio was 0.2 for chow, in which EPA and DHA constituted 1.0% and 2.2% of total FA, respectively. During the first 3 wk the mice were fed on the two different diets (chow or n-3 diet); afterward they all ate the standard chow. Each week a group was sacrificed to study the incorporation or depletion of n-3 PUFA. The three control groups were sacrificed in the first week (week 0) and at week 3 and week 6. At the end of the experimental period the mice were killed, and blood was collected by cardiac puncture with a syringe containing EDTA. The spleen and mesenteric lymph nodes were dissected.

Analysis of membrane lipids from erythrocytes. The EDTA-blood was centrifuged, and the erythrocytes were separated and washed with physiological saline after hemolysis in redistilled water (9). The membrane phospholipids were extracted and methylated as described previously (10). FAME were separated and quantified by GC (Hewlett-Packard Inc., Waldbronn, Germany) using the reported program (10). FAME were identified by comparing their retention times with those of standards (NU-Check-Prep Inc., Elysian, MN).

TABLE 1
FA Composition of Special Feed and Altromin (wt%)[a]

FA	Special feed	Altromin
12:0	—	0.3
14:0	5.4	0.7
15:0	0.5	0.1
16:0	18.2	14.6
16:1n-7	4.8	0.6
17:0	0.3	0.1
18:0	3.0	2.3
18:1n-9	29.3	18.5
18:1n-7	2.3	1.3
18:2n-6	3.6	50.0
20:0	0.3	0.2
18:3n-3	1.7	6.3
18:4n-3	1.3	—
20:1n-9	6.1	0.8
20:2n-6	0.3	—
22:0	0.1	0.2
20:4n-6	—	0.8
22:1n-11	9.0	—
20:4n-3	0.3	—
20:5n-3	2.2	1.0
24:1	0.7	—
22:5n-3	0.3	—
22:6 n-3	3.5	2.2
Σ n-6 PUFA	3.9	50.7
Σ n-3 PUFA	9.2	9.5
Ratio of n-3/n-6 PUFA	2.4	0.2

[a]Blanks mean not detected.

Analysis of phospholipids of leukocytes from the spleen and mesenteric lymph nodes. The spleen and mesenteric lymph nodes were removed aseptically and transferred to separate petri dishes containing sterile PBS. The spleen was mashed to free the cells from the tissue with the temple of a 1-mL syringe. The cell suspension was transferred to a 50-mL centrifugation glass. The same procedure was followed for the lymph nodes. The suspensions were centrifuged, and the supernatant was removed from the cells. The cells were incubated on ice for 5 min after adding 10 mL of 0.83% NH_4Cl. Then 30 mL of PBS was added, the supernatant was removed after centrifugation, and 2.5 mL of PBS was added to the cells. After transferring the solution to glass tubes and centrifuging, lipids were extracted from the pellet using the method mentioned above after adding the internal standard PC 15:0. The lipid extracts were separated by TLC, and phospholipids were scraped off and extracted. After methylation, the FAME were analyzed by GC.

Statistical analysis. Results are presented as means ± SD. Statistical analysis was performed using GraphPad PRISM version 3.02 (GraphPad software, San Diego, CA). Data were analyzed by one-way and two-way ANOVA. Differences were considered significant at $P < 0.05$.

RESULTS

Incorporation and depletion of PUFA in erythrocyte membranes. The composition of selected FA in erythrocyte membranes during the incorporation and depletion periods is shown in Table 2. The concentrations of EPA, DHA, and total n-3 FA

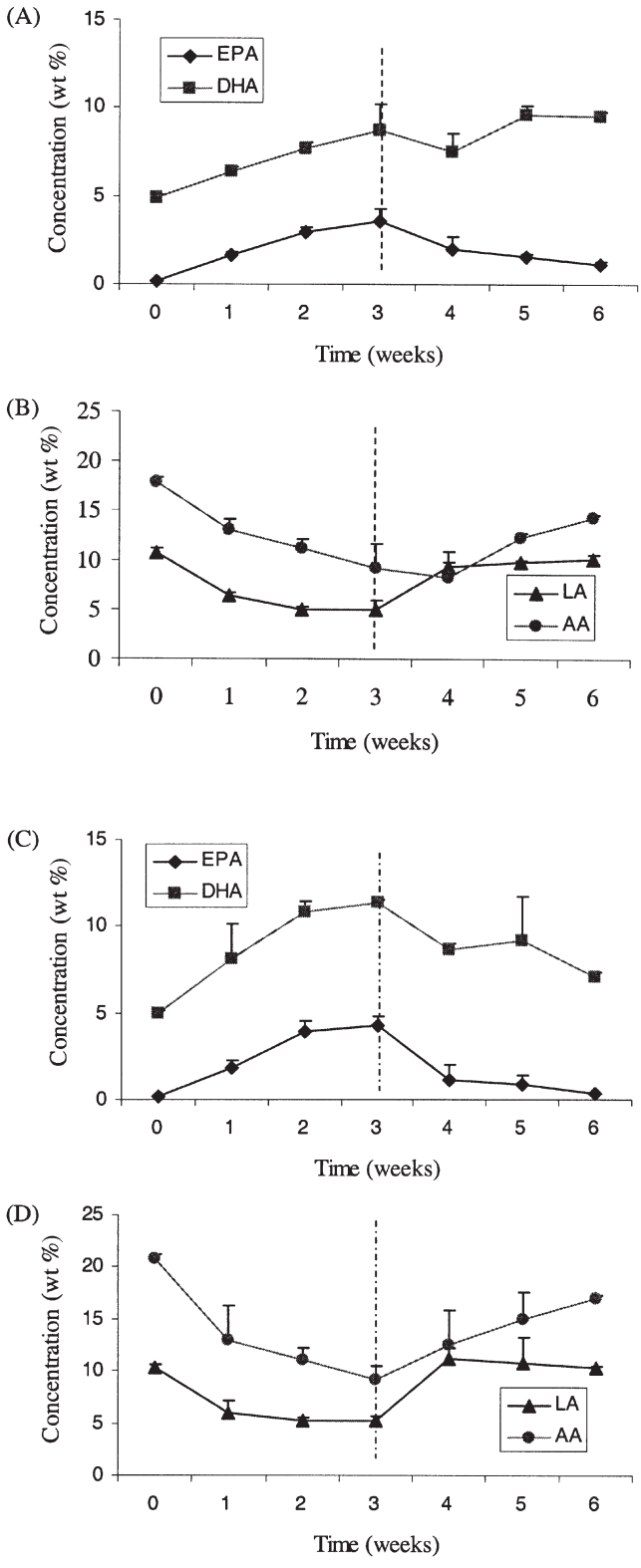

FIG. 1. The concentrations of (A) EPA and DHA in erythrocyte membrane lipids, (B) LA and AA in erythrocyte membrane lipids, (C) EPA and DHA in spleen leukocyte phospholipids, and (D) LA and AA in spleen leukocyte phospholipids, as a function of time. Values are shown for experimental groups fed special diet for the first 3 wk, then standard chow for the last 3 wk. Values are means + SD, $n = 4$.

TABLE 2
Incorporation and Depletion of n-3 FA (wt%) in Erythrocyte Membrane[a]

	Control	Incorporation period			Control	Depletion period			Control
FA	Week 0	Week 1	Week 2	Week 3	Week 3	Week 4	Week 5	Week 6	Week 6
18:2n-6	10.8 ± 0.4[a]	6.4 ± 0.3[b]	5.0 ± 0.3[b]	5.0 ± 0.9[b]	10.4 ± 1.1[a]	9.4 ± 1.4[a]	9.7 ± 0.2[a]	10.1 ± 0.4[a]	10.6 ± 0.2[a]
20:4n-6	17.8 ± 0.5[a]	13.1 ± 1.0[b,c]	11.2 ± 0.9[c,d]	9.1 ± 2.6[d,e]	15.7 ± 2.6[a,b]	8.3 ± 1.6[e,d]	12.2 ± 0.6[b,c,d]	14.3 ± 0.3[b,c]	18.2 ± 0.5[a]
20:5n-3	0.2 ± 0.1[a]	1.6 ± 0.2[b]	3.0 ± 0.3[c]	3.6 ± 0.6[c]	0.2 ± 0.1[a]	2.0 ± 0.7[b]	1.6 ± 0.2[b]	1.2 ± 0.1b	0.3 ± 0.0[a]
22:5n-3	0.8 ± 0.1	1.3 ± 0.6	1.2 ± 0.1	1.2 ± 0.2	1.3 ± 0.4	1.3 ± 0.3	1.4 ± 0.1	1.2 ± 0.1	0.8 ± 0.1
22:6n-3	4.9 ± 0.2[a]	6.3 ± 0.3[a,b]	7.7 ± 0.4[b]	8.7 ± 1.5[b,c]	4.4 ± 0.6[a]	7.5 ± 1.0[b]	9.6 ± 0.5[c]	9.5 ± 0.2[c]	5.4 ± 0.1[a]
Total n-6 PUFA	30.1 ± 1.0[a]	20.5 ± 1.2[b,d]	17.0 ± 1.1[b,c]	14.9 ± 3.6[c]	27.4 ± 3.7[a]	18.5 ± 1.9[b,c,d]	23.3 ± 0.5[d,e]	25.7 ± 0.2[a,e]	30.1 ± 0.6[a]
Total n-3 PUFA	6.2 ± 0.3[a]	10.4 ± 1.0[b]	13.0 ± 0.6[c]	14.5 ± 2.0[c]	6.6 ± 0.2[a]	12.1 ± 1.9[b,c]	13.3 ± 0.7[c]	12.6 ± 0.4[b,c]	7.0 ± 0.2[a]
Ratio of n-3/n-6 PUFA	0.2 ± 0.0[a]	0.5 ± 0.0[b]	0.8 ± 0.1[c]	1.0 ± 0.2[d]	0.2 ± 0.0[a]	0.7 ± 0.1[b,c]	0.6 ± 0.0[b]	0.5 ± 0.0[b]	0.2 ± 0.0[a]

[a]Values are means ± SD, $n = 4$. Values in the same row with no superscript letter in common are significantly different ($P < 0.05$).

increased linearly during the incorporation period (Fig. 1a). During the 3-wk depletion period, however, the concentration of DHA remained almost constant. The concentration of EPA decreased significantly during the first week of depletion, followed by slow but continued reduction during the next 2 wk. The level of EPA was still significantly higher than the background level after 3 wk of depletion.

The concentrations of AA and LA declined throughout the incorporation period, and the level of LA changed very fast with changes of dietary lipids (Fig. 1b). In both cases, there was no significant difference between week 2 and week 3. During the depletion period, the concentration of n-6 PUFA increased; LA increased during the first week and already reached the background level after 1 wk, whereas AA increased after the first week and did not reach the background level after 3 wk.

Incorporation and depletion of PUFA in phospholipids of spleen and lymph leukocyte. We studied the FA of phospholipids from both spleen leukocytes and mesenteric lymph leukocytes during the whole feeding period. The composition of selected FA of phospholipids from spleen leukocytes during the whole feeding period is listed in Table 3. Both EPA and DHA of phospholipids from spleen leukocytes increased as a function of feeding time during the first 3 wk (Fig. 1c). Similar results were obtained for lymph node leukocytes (data not shown). Dissimilar to the erythrocyte phospholipids, the concentration of both EPA and DHA decreased during the depletion period, with the most significant decrease during the first week, but they did not reach the level measured at the beginning of the feeding period.

The concentration of both AA and LA declined during the first 3 wk, and increased again after changing back to the normal chow diet (Fig. 1d).

DISCUSSION

The ratio of n-3/n-6 PUFA in both erythrocyte and leukocyte was similar to that in the chow when the feeding experiment was started. Even though the concentrations of EPA and DHA of the special feed were only 2 and 1.5 times, respectively, higher than that in the chow, the ratio of n-3/n-6 PUFA in the special feed was 10 times higher than that in the chow because of the high concentration of LA in the chow. Therefore the significant increase of EPA and DHA in both erythrocytes and leukocytes indicates that the ratio of n-3/n-6 PUFA is more important than the absolute amount of n-3 FA.

The ratio of n-3/n-6 PUFA in both erythrocyte and leukocyte increased when mice got the special feed. However, the incorporation of EPA and DHA in erythrocytes was different from that in leukocytes. Because of the linear increase in the incorporation of EPA and DHA into erythrocytes (Fig. 1), one may expect that more EPA and DHA will be incorporated into erythrocytes if the mice get the special diet for a longer period, corresponding to the relative long lifespan of erythrocytes (3 mon). The concentrations of AA and LA in erythrocytes changed in the opposite ways

TABLE 3
Incorporation and Depletion of n-3 FA (wt%) in the Phospholipids of Spleen Leukocyte[a]

	Control	Incorporation period			Control	Depletion period			Control
FA	Week 0	Week 1	Week 2	Week 3	Week 3	Week 4	Week 5	Week 6	Week 6
18:2n-6	10.3 ± 0.3[a]	6.0 ± 1.1[b]	5.2 ± 0.4[b]	5.3 ± 0.4[b]	10.8 ± 0.4[a]	11.2 ± 1.0[a]	10.7 ± 2.5[a]	10.4 ± 0.1[a]	10.4 ± 0.2[a]
20:4n-6	20.7 ± 0.6[a]	12.9 ± 3.3[b,c]	11.1 ± 1.1[c]	9.1 ± 1.3[c]	20.1 ± 0.5[a]	12.6 ± 3.3[b,c]	15.0 ± 2.6[b,c,d]	17.0 ± 0.4[a,b,d]	19.6 ± 0.3[a,d]
20:5n-3	0.1 ± 0.0[a]	1.9 ± 0.4[b]	3.9 ± 0.7[c]	4.3 ± 0.5[c]	0.1 ± 0.1[a]	1.2 ± 0.9[a,b]	0.9 ± 0.5[a,b]	0.3 ± 0.1[a]	0.2 ± 0.0[a]
22:5n-3	1.6 ± 0.0[a,d]	2.7 ± 0.6[b]	4.1 ± 0.3[c]	3.9 ± 0.3[c]	1.3 ± 0.1[a]	2.3 ± 0.4[b,d]	1.5 ± 0.6[a,d]	1.4 ± 0.1[a,d]	1.5 ± 0.2[a,d]
22:6n-3	4.9 ± 0.2[a]	8.1 ± 2.0[b,c]	10.8 ± 0.6[c,d]	11.4 ± 0.1[d]	4.8 ± 0.3[a]	8.7 ± 0.3[b,c]	9.2 ± 2.6[b,c,d]	7.0 ± 0.3[a,b]	4.8 ± 0.3[a]
Total n-6 PUFA	33.7 ± 0.7[a]	20.4 ± 4.3[b,c]	17.7 ± 1.2[c]	15.8 ± 1.7[c]	33.6 ± 1.0[a]	26.2 ± 2.6[b]	28.0 ± 5.0[a]	29.8 ± 0.4[a]	32.6 ± 0.6[a]
Total n-3 PUFA	6.9 ± 0.2[a]	13.6 ± 2.9[b]	19.6 ± 1.2[c]	20.7 ± 1.0[c]	6.6 ± 0.3[a]	12.7 ± 1.3[b]	12.0 ± 2.2[b]	9.3 ± 0.2[a]	6.8 ± 0.1[a]
Ratio of n-3/n-6 PUFA	0.2 ± 0.0[a,d]	0.7 ± 0.1[b]	1.1 ± 0.1[c]	1.3 ± 0.2[c]	0.2 ± 0.0[a]	0.5 ± 0.1[b,d]	0.4 ± 0.1[d]	0.3 ± 0.0[a,d]	0.2 ± 0.0[a,d]

[a]Values are means ± SD, n = 4. Values in the same row with no superscript letter in common are significantly different ($P < 0.05$).

in the erythrocytes, reflecting the metabolic competition between both PUFA families.

Yaqoob *et al.* (11) studied plasma phospholipids and blood mononuclear cell FA composition of people taking fish oil capsules for 12 wk, and they found that the concentration of EPA and DHA in both plasma phospholipids and mononuclear cell lipids reached the highest level at week 4, and there was no significant differences during the following 8 wk. There was no information about how the FA composition changed during the first 4 wk in their study. In the present study we found that the concentration of EPA and DHA in leukocyte phospholipids also increased linearly in the first 2 wk; thereafter a slower incorporation was observed in mice. This could be due to the fast turnover time of leukocytes in comparison with erythrocytes. These results may have important implications for clinical nutrition, in addition to contributing to a better understanding of lipid metabolism.

In conclusion, the present study shows that dietary lipids strongly influence the FA composition of erythrocyte membranes and leukocyte phospholipids in mice, but the incorporation rate of n-3 FA in erythrocytes and leukocytes are different. The rate of incorporation is faster than that of depletion for n-3 PUFA. More n-3 PUFA can be incorporated into leukocytes in comparison with erythrocytes. The ratio of n-3/n-6 PUFA is more important than the amount of n-3 FA in changing the FA compositions of membrane lipids, but more study is required to support this conclusion.

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Partial Prevention of Hepatic Lipid Alterations in Nude Mice by Neonatal Thymulin Gene Therapy

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ABSTRACT: During adult life athymic (nude) male mice display not only a severe T-cell–related immunodeficiency but also endocrine imbalances and a moderate hyperglycemia. We studied the impact of congenital athymia on hepatic lipid composition and also assessed the ability of neonatal thymulin gene therapy to prevent the effects of athymia. We constructed a recombinant adenoviral vector, RAd-metFTS, expressing a synthetic DNA sequence encoding met-FTS, an analog of the thymic peptide facteur thymique sérique (FTS), whose Zn-bound biologically active form is known as thymulin. On postnatal day 1–2 homozygous (nu/nu) nude and heterozygous (nu/+) mice were injected with 10^8 pfu of RAd-metFTS or RAd-βgal (control vector) intramuscularly. The animals were processed at 52 d of age. Serum thymulin, glycemia, hepatic phospholipid FA composition and free and esterified cholesterol were determined. Adult homozygous male nudes were significantly ($P < 0.01$) hyperglycemic when compared with their heterozygous counterparts (2.04 vs. 1.40 g/L, respectively). The relative percentage of 16:0, 18:1n-9, and 18:1n-7 FA was lower, whereas that of 18:0, 20:4n-6, and 22:6n-3 FA was higher, in hepatic phospholipid (PL) of nu/nu animals as compared with their nu/+ counterparts. Some of these alterations, such as that in the relative content of 22:6n-3 in liver PL and the unsaturation index, were completely or partially prevented by neonatal thymulin gene therapy. We conclude that the thymus influences lipid metabolism and that thymulin is involved in this modulatory activity.

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During adult life, congenitally athymic (nude) mice display, in addition to a severe immunodeficiency, multiple endocrine alterations (1–4) that are reflected by deficits in some hormone-dependent functions such as reproduction (5,6). There is evidence that these hormone-dependent functional alterations may also include glucose homeostasis. Thus, it has been reported that after 1 mon of age, nude BALB/c mice develop spontaneous hyperglycemia and impaired glucose tolerance (7,8). Furthermore, these animals show peripheral insulin insensitivity with normal pancreatic β-cell reserve and normal lean body mass (9). Assessment of pancreatic islet cell populations in hyperglycemic nudes revealed an increase in the D-cell (somatostatin-producing) population. Also, somatostatin content in pancreatic tissue was higher in the athymic nudes as compared with heterozygous sex- and age-matched counterparts (10). Adult thymectomy in Wistar rats was reported to increase circulating insulin levels without significant changes in blood glucose (11).

Although we are unaware of documented studies characterizing lipid metabolism in athymic nude mice as compared with heterozygous counterparts, there are studies pointing to a modulatory activity of thymic factors on lipid metabolism. A thymic protein factor was reported to reduce serum cholesterol levels in rats, increase LDL catabolism, and inhibit the activity of hepatic HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis (12–14).

In the present study we document that adult athymic nude mice display a number of differences in hepatic lipid composition relative to heterozygous counterparts. We also report that implementation of neonatal gene therapy with a synthetic gene for the thymic metallopeptide thymulin prevents the emergence of some of these differences in adult athymic nudes.

MATERIALS AND METHODS

Adenoviral vector RAd-metFTS. We constructed a DNA sequence, 5'-ATGCAAGCCAAATCTCAAGGTGG-ATCCAA-CTAGTAG-3', coding for the peptide met-QAKSQGGSN (met-FTS), an analog of the thymic peptide facteur thymique sérique (FTS), whose biologically active form is called thymulin. Here, this DNA sequence is referred to as synthetic gene for thymulin. Subsequently, a recombinant adenoviral (RAd) vector harboring the synthetic gene for thymulin was constructed by a variant of the two-plasmid method (15) employing the AdMax® plasmid kit (Microbix, Hamilton, Canada) as previously described (16). The newly generated vector, termed RAd-metFTS, had the met-FTS synthetic gene under the control of the mouse cytomegalovirus promoter (Fig. 1). The virus was rescued from HEK293 cell lysates and it was plaque purified. The virus was further purified by ultracentrifugation in CsCl gradient. Final virus stocks were titrated by a serial dilution plaque assay.

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Abbreviations: FTS, facteur thymique sérique; GH, growth hormone; LH, luteinizing hormone; pfu, plaque-forming units; PL, phospholipid; PRL, prolactin; RAd, recombinant adenovirus; TSH, thyrotropin; Tx, thymectomized;

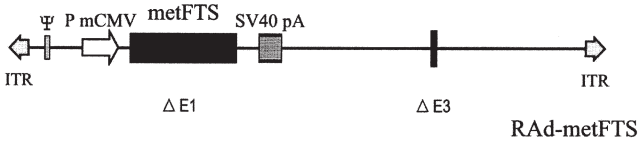

FIG. 1. Diagrammatic representation of the RAd-metFTS vector. PmCMV, mouse cytomegalovirus promoter; metFTS, synthetic gene for met-FTS; frt, recognition element for the yeast FLP recombinase; ITR, inverted terminal repeat; ΔE1 and ΔE3, deletions in the Ad5 genome; SV40, simian virus 40 polyadenylation signal; Ψ, packaging signal.

RAd-βgal (control vector). This RAd was kindly provided by Dr. Michel Perricaudet, Institut Gustave Roussy, CNRS, Villejuif, France. In this vector, the E1 genomic region has been replaced by an expression cassette containing the *E. coli* lac Z reporter gene under the control of the Rous sarcoma virus long terminal repeat. The vector was expanded in HEK 293 cells and purified and titrated as indicated for RAd-FTS.

Animals and in vivo procedures. The male offspring from NIH homozygous (nu/nu) nude male and heterozygous (nu/+) female mice were used in the present study. All mice were maintained on a γ-irradiated chow diet and sterilized water. Animals had free access to food and water and were kept at 22ºC with a light/dark cycle of 12/12 h. All experiments on animals were done in conformity with the Guidelines on Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare (17).

At postnatal day 1 or 2, each pup received a single bilateral intramuscular (hindlegs) injection of 10^8 plaque-forming units (pfu) RAd-metFTS or RAd-βgal in 10 µL PBS (5 µL per side). At 5–6 d of age heterozygous and homozygous pups could be easily identified by the presence of absence of hair, respectively. Pups of both phenotypes were kept together in each litter. On postnatal day 51–52 mice were bled from the retroorbital plexus and immediately sacrificed by cervical dislocation. Livers were removed and processed as indicated below.

Lipid extraction and analysis. Plasma glucose concentration was determined by the glucose oxidase method. Hepatic lipids were extracted with chloroform/methanol (2:1 vol/vol) (18). An aliquot was used to separate and quantitate free and esterified cholesterol by TLC as described elsewhere (19). The PL fraction was separated from the rest of the organic phase by a microcolumn chromatography method described by Hannahan *et al.* (20). Lipid phosphorous (21) was determined. FAME from PL were prepared by methylation with BF_3/methanol according to the method of Morrison and Smith (22). The analyses were performed using a Shimadzu 9A gas chromatograph (Tokyo, Japan) fitted with a Omegawax 250 fused-silica column, 30 m × 0.25 mm, with 0.25 µm phase (Supelco, Bellafonte, PA). After 1 min initial hold, the oven temperature was programmed to increase from 185 to 220°C at 3°C/min. Peaks were identified by comparing the retention times with those from a mixture of methyl ester standards (Sigma Chemical Co., St. Louis, MO).

Thymulin bioassay. Biologically active thymulin was measured in serum by a rosette bioassay described in detail elsewhere (23). This method is based on the ability of thymulin to restore the inhibitory effect of azathioprine on rosette formation in spleen cells from thymectomized (Tx) mice. The inhibitory activity of samples was compared with that of a standard curve using synthetic thymulin (a kind gift of Dr. Peter Heinklein, Humboldt University, Berlin, Germany). Serum values were expressed as fg/mL bioactive thymulin.

Statistical analysis. Statistical analyses were performed by means of ANOVA using GB-STAT Professional Statistics and Graphics Version 4.0 (Dynamic Microsystems Inc., Silver Springs, MD). The Tukey t-test was used for comparisons between means. Statistical significance was assigned to $P < 0.05$.

RESULTS

Effects of congenital athymia on plasma levels of glucose. As expected, 3-mon-old congenitally athymic nude mice showed a significant ($P < 0.01$) hyperglycemia. Thus, nu/+ and nu/nu mice (10 animals per group) had a glycemia of 1.40 ± 0.18 and 2.04 ± 0.63 (g/L; mean ± SD), respectively.

Restorative effect of thymulin gene therapy on serum thymulin in nude mice. Neonatal intramuscular injection of RAd-metFTS, but not RAd-βgal, increased the circulating levels of biologically active thymulin in both heterozygous and homozygous nude mice tested at 51–52 d of age (Table 1). At 13 d of age the RAd-metFTS-treated mice already had increased serum thymulin levels (Table 1). In adult mice, thymectomized (Tx) after puberty, and which have undetectable levels of serum thymulin, intramuscular injection of RAd-βgal failed to induce detectable serum thymulin levels, whereas Tx animals injected with RAd-metFTS showed a progressive increase in serum thymulin (data not shown). However, lipid changes were not studied in Tx because Tx

TABLE 1
Effect of Neonatal RAd-metFTS Intramuscular Injection on Serum Levels of Thymulin in Heterozygous and Homozygous Nude Mice[a]

	RAd-βgal		RAd-metFTS	
Age	13 d	51–52 d	13 d	51–52 d
nu/+	ND	103 ± 15 (5)	ND	512 ± 140 (5) ($P < 0.05$)[b]
nu/nu	4 ± 9 (3)	34 ± 9 (5)	69 ± 12 (3)[b]	288 ± 32 (8) ($P < 0.01$)

[a]Serum thymulin values (fg/mL) are expressed as mean ± SEM; n values are given in parentheses.
[b]*P* values indicate significance of differences between RAd-βgal- and RAd-metFTS-injected counterparts at the same postinjection day. ND, not determined.

TABLE 2
Effect of Thymulin Gene Therapy on FA Composition of Hepatic Phospholipids in Male Nude Mice[a]

FA	nu/+; RAd-βgal (n = 5)	nu/nu; RAd-βgal (n = 5)	nu/nu; RAd-FTS (n = 4)	nu/+; RAd-FTS (n = 8)
16:0	25.43 ± 0.78^{NS}	23.94 ± 0.61^{NS}	25.64 ± 0.48^{NS}	25.26 ± 2.73^{NS}
16:1n-7	1.17 ± 0.12^{a}	0.18 ± 0.13^{b}	0.35 ± 0.44^{b}	1.15 ± 0.51^{a}
18:0	15.90 ± 1.19^{a}	18.19 ± 0.85^{b}	18.09 ± 0.96^{b}	15.34 ± 0.96^{a}
18:1n-9	10.62 ± 2.18^{a}	8.35 ± 0.97^{b}	9.40 ± 0.36^{a,b,d}	12.08 ± 1.35^{a,c}
18:1n-7	2.67 ± 0.53^{a}	1.42 ± 0.21^{b}	1.72 ± 0.32^{b}	2.60 ± 0.49^{a}
18:2n-6	17.37 ± 1.27^{NS}	19.17 ± 1.18^{NS}	19.40 ± 0.28^{NS}	18.02 ± 1.50^{NS}
20:3n-6	2.54 ± 0.45^{NS}	2.15 ± 0.13^{NS}	1.98 ± 0.40^{NS}	2.68 ± 0.34^{NS}
20:4n-6	18.79 ± 1.39^{a,c}	20.23 ± 0.84^{a}	18.10 ± 0.29^{b,c}	17.55 ± 1.32^{b,c}
22:6n-3	5.52 ± 0.15^{a}	6.47 ± 0.59^{b}	5.33 ± 0.30^{a}	5.32 ± 0.53^{a}
UI	165.10 ± 3.90^{a}	174.20 ± 4.70^{b}	160.60 ± 3.60^{a}	162.10 ± 8.40^{a}

[a]Only main FA were considered. UI = unsaturation index = sum of percentage levels × number of double bonds. Values are given as means ± SD. Values without common superscript letters are significantly different ($P < 0.05$). NS, not significant.

performed in adult rodents has no immune or endocrine consequences, and therefore changes in liver lipid profiles are unlikely. On the other hand neonatal Tx in mice is associated with a high mortality.

Effect of thymulin gene therapy on hepatic lipid composition in nude mice. FA composition of PL from nu/nu and nu/+ livers are shown in Table 2. Significant differences were observed in palmitoleic (16:1n-7), oleic (18:1n-9), vaccenic (18:1n-7), stearic (18:0), arachidonic (20:4n-6), and docosahexaenoic (22:6n-3) acids.

The relative percentage of 16:0, 18:1n-9, and 18:1n-7 was lower, whereas that of 18:0, 20:4n-6, and 22:6n-3 was higher, in hepatic phospholipid of nu/nu animals as compared with nu/+ counterparts. The higher percentage of arachidonic and docosahexaenoic acid accounted for the higher unsaturation index (UI) found in nu/nu mice relative to heterozygous animals (Table 2).

The content of monounsaturated and saturated FA was unaffected by thymulin gene therapy, but the treatment prevented the increase of 20:4 and 22:6 observed in the control nudes relative to control heterozygous counterparts. The changes induced by athymia in the UI were also prevented by neonatal thymulin gene therapy. Other less abundant (<0.6%) FA were detected in our study, but none of them was significantly affected by athymia or thymulin gene therapy (data not shown). Although neither congenital athymia nor thymulin gene therapy significantly affected free, esterified, and total liver cholesterol or PL:cholesterol ratio, near significant differences were observed in some cases (Fig. 2).

DISCUSSION

The present results confirm previous studies reporting that adult nude mice develop a moderate hyperglycemia (7,8). Our data additionally document, for the first time to our knowledge,

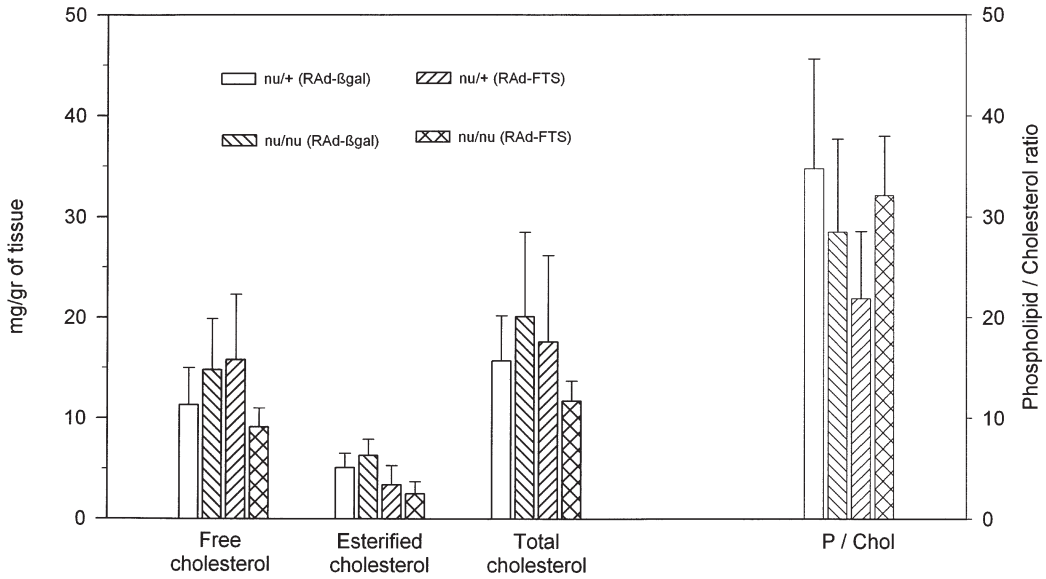

FIG. 2. Effect of thymulin gene therapy on hepatic cholesterol composition in nude mice. Male 51–52-d-old mice were used. Data are expressed as mean ± SD. Although no significant differences were found among groups, trends toward a difference were detected for some of the parameters studied.

that adult male nudes have a number of alterations in their hepatic FA composition profiles. Because the biosynthesis of polyenoic acids is largely modified by a wide variety of hormonal conditions (24,25) and congenital athymia is associated with alterations in the balance of several hormones (6,26), our finding of altered FA composition of PL in the liver of nude mice was not unexpected. On the other hand, liver cholesterol metabolism does not seem to be affected by athymia, although this might not be completely so in view of the trend toward a change observed for athymic mice for some of the cholesterol variables measured.

The ability of neonatal thymulin gene therapy to prevent some of the changes induced by athymia in hepatic FA composition of PL may be explained by a possible restorative activity of thymulin on the endocrine balance. This hypothesis is supported by the evidence showing that athymia and thymulin administration have opposite effects. Thus, congenital athymia is associated with reduced levels of circulating and pituitary gonadotropins in female nude mice (6), whereas in male nudes thyrotropin (TSH), prolactin (PRL), growth hormone (GH), and gonadotropin responses to immobilization and cold stress are reduced, as are serum basal levels of the same hormones (2–4). Conversely, thymulin has been shown to stimulate luteinizing hormone (LH) release from perifused rat pituitaries (27) and corticotropin release from incubated rat pituitary fragments (28). Thymulin also stimulates gonadotropin, PRL, TSH, and GH release in dispersed rat pituitary cells at doses ranging from 10^{-8} to 10^{-5} M, an effect that declines with the age of the pituitary donors (29–31).

Although a direct effect of thymulin at hepatic level cannot be ruled out, in pilot studies (unpublished) in which A549 cell cultures were exposed to different doses of synthetic thymulin, we could not detect significant changes of FA composition in total PL. It should be also mentioned that although there are no documented studies on the effect of thymulin on lymphocyte lipid composition, it is not unlikely that thymulin may modulate, by a direct action, lipid metabolism in thymulin-responsive T-lymphocytes.

Considering that liver desaturases are sensitive to endocrine factors (25), the possibility exists that thymulin may have a modulatory action (direct or indirect) on the activity and/or expression of these enzymes.

Our results also indicate that although some of the changes induced by athymia in hepatic FA composition are prevented by thymulin gene therapy, others are not. The former case corresponds to the relative content of 22:6n-3 in liver PL and UI, whereas the latter case corresponds to the relative content of 16:1 and 18:0. In general, treatment of heterozygous nudes with RAd-metFTS did not affect hepatic FA composition despite the fact that the viral vector significantly increased serum thymulin levels in both homozygous and heterozygous nudes. This observation suggests that thymulin may act as a permissive molecule that allows an optimal performance of primary effectors on lipid metabolism and/or the endocrine balance.

The present results point to the thymus as an organ that, acting during early life, influences lipid metabolism in adult life, and also to thymulin as a mediator of part of this modulatory activity.

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Intrasample Variability of Intramyocellular Triacylglycerol

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ABSTRACT: Intrasample variability of intramyocellular triacylglycerol (imcTG) in the skeletal muscle of rats has been examined. Aliquoting after homogenization of muscle samples reduced imcTG variability considerably compared with aliquoting before homogenization. The results suggested that skeletal muscle samples be homogenized before aliquoting in order to reduce imcTG variability.

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Intramyocellular triacylglycerol (imcTG) is an indispensable energy source for muscle work (1). Measuring imcTG has been used as a means to quantify the utilization of imcTG, but there is a high degree of difficulty in detecting changes in imcTG pool size. Thus, imcTG utilization remains controversial (2). The difficulty is mainly contributed by the inherently high variability of imcTG content itself (3–6). Although not preferred (7), the method has the advantage of simplicity. It is still widely used in research.

It is a common practice to take an aliquot from a muscle sample for imcTG determination. However, if intrasample variability (IV) is high, the result may not represent the entire sample and thus introduces errors. Therefore, it is necessary to examine IV of imcTG measurement, which so far has not been studied adequately. To date, the majority of imcTG variability studies are focused on intersample or interindividual variability. Recently, Wendling *et al.* (4) evaluated the variability between two aliquots of pulverized powder from same muscle samples, an approach potentially useful for reducing imcTG variability. Unfortunately, the study did not investigate this issue in more detail.

In the present studies, we compared two different approaches to processing muscle samples for the measurement of imcTG, namely, muscle samples are homogenized either before or after being aliquoted for imcTG analysis. Variability of non-esterified FA was also determined and compared to that of imcTG.

MATERIALS AND METHODS

Animals and protocol. Sprague-Dawley male rats (n = 10, 450–500 g body wt) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The animals were kept in a room with 12 h/12 h light/dark cycles and stable temperature and humidity. They were studied after an overnight fast, awake. The study protocols were approved by Mayo Institutional Animal Care and Use Committee.

In order to monitor potential lipid contamination by extracellular lipids and imcTG stability during sample storage and processing, the imcTG pool was first labeled by intravenous infusion of ^{14}C-glycerol at 0.1 μCi/kg/min via a tail vein for 120 min while rats rested calmly in a metabolic cage (8). At the end, the animals were anesthetized by pentobarbital injection (50 mg/kg) and euthanized by open heart bleeding in order to reduce blood in tissues. Red gastrocnemius and tibialis anterior muscles were swiftly excised and divided into two approximately equal portions (~200 mg each). Each portion was cut into small pieces (~20 mg, so that they are chilled into the core instantaneously), labeled, and saved in liquid nitrogen. Visible fat and connective tissues were removed rapidly but no detailed dissection was performed until sample processing and lipid extraction.

Prior to lipid extraction, the frozen small muscle pieces from each portion were individually thawed on ice and dissected under a stereo microscope (10X-40X, Motic Microscopes) on ice to remove fat cells (this would have been impossible if cold tongs were used to clamp-freeze the samples as it crushes the fat cells, (9)). Upon completion, each piece was set on dry ice. When all pieces from one portion (set A) were completed, they were randomly divided into two aliquots of similar size (~100 mg each) (pair A) and homogenized separately in chloroform-methanol (2:1), kept on ice (aliquoting before homogenization, ABH). All the pieces from the other portion (set B) were homogenized together before being divided into two aliquots of similar size (~100 mg each, pair B) (aliquoting after homogenization, AAH). All of the homogenates were extracted according to Folch (10), and imcTG and NEFA were purified by TLC in a solvent system of hexane:ether:acetic acid (70:30:1). The purified imcTG and NEFA were quantified enzymatically (11). One aliquot of the imcTG-glycerol from pair A was also used to count for ^{14}C activity in order to determine specific activity.

IV of imcTG and NEFA using the ABH approach was determined from pair A and expressed as the absolute difference in imcTG or NEFA content ($|\Delta|$) between the two aliquots divided by their average imcTG or NEFA content (M):

$$\text{IV} = |\Delta|/M \times 100\% \quad [1]$$

IV of imcTG specific activity was calculated in a similar way. Processing variability (PV) for imcTG using the AAH approach was determined from pair B using an equation analogous to Equation 1.

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Abbreviations: AAH, aliquoting after homogenization; ABH, aliquoting before homogenization; imcTG, intramyocellular triacylglycerol; IV, intrasample variability; PV, processing variability.

Statistics. Comparisons of imcTG contents within pairs, of variability between pairs, and between IV of imcTG and NEFA were made using paired Student's t-test.

RESULTS AND DISCUSSION

imcTG variability. Table 1 shows that imcTG contents for the two aliquots from gastrocnemius and tibialis anterior using the ABH approach were similar ($P > 0.2$). The IV were high: 22.4 ± 7.0% for gastrocnemius and 22.7 ± 6.1% for tibialis anterior. By comparison, the PV were 4 times lower ($P < 0.01$) than the IV: 5.9 ± 0.9% for gastrocnemius and 6.5 ± 1.4% for tibialis anterior. This indicated that the sample processing and analysis were reproducible. Thus, most (>75%) of the IV was due to muscle heterogeneity. The observed IV was similar to the intersample (4) and interindividual variability (12–14) reported in some studies, but lower than those reported in other studies (5,15,16).

IV at this level can cause significant errors in imcTG determination. For example, if aliquot 1 or aliquot 2 were used to measure imcTG content of the entire sample, the magnitude of potential errors is ~22%. This suggested that the lower detection limit for changes in imcTG is 22% (15,16). In order to detect a difference equal to the variability, a sample size of 17 is required at $\alpha = 0.05$ level with a statistical power of 0.80 (18). Many studies do not use samples of this size, and this could have contributed to the failures of detecting imcTG utilization. To detect imcTG changes of 1.5 times or twice the variability, the sample size is reduced to $n = 9$ or $n = 6$, respectively, the size more commonly used in research. However, in such cases, the lower detection limits increase to 37.5% or 50%, respectively. Such magnitudes of changes in imcTG content are relatively rare. Thus, high imcTG variability appears to be the main reason why many studies failed to detect changes in imcTG (2,5,17).

It is important to note that the observed high variability of imcTG using the ABH approach was not caused by lipid contamination, as demonstrated by the consistent imcTG ^{14}C-specific activity between the two aliquots. This would have been impossible if the samples were contaminated by extracellular lipids (adipocytes), which dramatically change the specific activity of imcTG due to their distinct metabolism. But, the variability (IV) of imcTG ^{14}C-specific activity obtained using ABH was also high, and comparable ($P > 0.05$) to that of imcTG content (Table 1). As expected, the variability (PV) of imcTG ^{14}C-specific activity obtained using the AAH approach was reduced significantly ($P < 0.05$, values not shown). The high IV when using the ABH approach was not due to imcTG autolipolysis either during sample processing, because NEFA content for these samples had low variability, which is less likely if uncontrolled autolipolysis was present.

imcTG variability was reduced to approximately 6% by using the AAH approach. Pulverization of freeze-dried samples appeared to have a similar degree of the same effect (4). Together, these observations suggested that, for the purpose of imcTG measurement, the AAH approach helps reduce the variability significantly by mixing the entire sample, thereby eliminating intrasample heterogeneity. Thus, the result should be more representative of the sample. Apparently, however, this approach does not apply in some cases, such as if fiber type–specific information is sought (19). Lipid contamination, if present, may render the approach less useful because it will spread the contaminants to the entire sample. Nonetheless, using the ABH approach does not solve this issue either. Therefore, removing lipid contaminants is a prerequisite in all cases.

NEFA variability. By comparison, even using the ABH approach, IV for NEFA was significantly lower than that for imcTG ($P < 0.05$) for both gastrocnemius (12.2 ± 2.1%) and tibialis anterior (11.8 ± 3.4%). However, by using the AAH approach, the variability was not significantly reduced (not shown). Intersample variability of NEFA is also lower than that for imcTG. For gastrocnemius, the average coefficient of variation (a measure of intersample variability) for the two aliquots was 9% and 45% for NEFA and imcTG, respectively. The corresponding values for tibialis anterior were 14% and 27%. These data suggest that muscle heterogeneity mainly affects imcTG with smaller effect on NEFA. Because imcTG variability is mainly caused by nonhomogeneous fiber type distribu-

TABLE 1
Intrasample imcTG Variability for Rat Skeletal Muscle Using ABH vs. AAH[a]

	Gastrocnemius			Tibialis anterior		
	Aliquot 1	Aliquot 2	Average	Aliquot 1	Aliquot 2	Average
ABH (pair A)	1.95 ± 0.28	1.70 ± 0.24	1.82 ± 0.24	2.14 ± 0.23	2.44 ± 0.15	2.29 ± 0.18
IV	22.4 ± 7.0%			22.7 ± 6.1%		
SA, dpm/μM[e]	537 ± 74	536 ± 31	537 ± 52	696 ± 91	699 ± 110	697 ± 96
IV[b]	16.9 ± 4.7%			14.4 ± 3.9%		
AAH (pair B)	1.92 ± 0.27	1.86 ± 0.25	1.90 ± 0.26	2.23 ± 0.19	2.38 ± 0.20	2.31 ± 0.18
PV[c]	5.9 ± 0.9%[d]			6.5 ± 1.4%[d]		

[a]Values are mean ± SEM ($n = 10$). imcTG content is in μmol/g wet muscle. There were no differences in imcTG content within or between pair A and pair B. imcTG, Intramyocellular tricylglycerol; ABH, aliquoting before homogenization; AAH, aliquoting after homogenization; IV, intrasample variability; PV, processing variability.
[b]IV is for pair A, processed using ABH.
[c]PV is for pair B, processed using AAH. IV and PV are the averages of individual within-pair variability, not calculated from the two averages.
[d]Significantly lower than IV of pair A ($P < 0.01$).
[e]SA, ^{14}C-specific activity of imcTG for pair A (its IV calculation is analogous to Equation 1).

tion (19,20), the observation indicated that non-esterified FA exist more evenly among different fiber types than imcTG, which is more concentrated in oxidative fiber types (I > IIa > IIb).

CONCLUSION

For imcTG analysis, homogenization before aliquoting can help reduce IV significantly. The relatively low variability for non-esterified FA suggest that the metabolite is more homogeneously distributed than triacylglycerol among different muscle types.

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Desaturation Indices in Liver, Muscle, and Bone of Growing Male and Female Mice Fed *trans*-10,*cis*-12 Conjugated Linoleic Acid

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ABSTRACT: *trans*-10,*cis*-12-CLA (*t*10,*c*12-CLA) inhibits lipid deposition in adipose tissue of many species, but it also enhances lipid deposition in liver. We evaluated effects of dietary *t*10,*c*12-CLA content and gender on carcass composition, FA profile of selected tissues, and expression of FA synthase (FAS) and stearoyl-CoA desaturase-1 (SCD) mRNA in adipose tissue. Male and female (63 of each) CD-1 mice were assigned a diet containing 0.0, 0.15, or 0.30% *t*10,*c*12-CLA at 4 wk of age. Seven mice per dietary group within gender were sacrificed after 2, 4, or 6 wk. The CLA isomer caused dose-dependent reductions in dry carcass weight and fat content, without altering protein content, but carcass fat and epididymal fat pad weights of males were reduced to a greater extent than carcass fat and inguinal fat pad weights of females. FAS and SCD mRNA in adipose tissue was more abundant in females than males, but expression in both genders decreased as the *t*10,*c*12-CLA content of the diet increased. Although the weight of gastrocnemius muscle was not influenced by diet, total FA content of the muscle of both genders decreased in response to dietary *t*10,*c*12-CLA content. Femur weight of male mice increased as the *t*10,*c*12-CLA content of the diet increased, but the weight increase was associated with a reduction in total FA content. The Δ9 desaturation indices for muscle and femur suggested a linear reduction in SCD activity, whereas Δ9 indices for liver indicated linear enhancement of SCD activity. Overall, results suggested that growing male mice were more susceptible than females to *t*10,*c*12-CLA inhibition of lipid deposition.

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Alterations in lipid metabolism attributed to *trans*-10,*cis*-12-CLA (*t*10,*c*12-CLA) include reduced deposition of lipid in body fat depots, increased deposition of lipid in liver, and reduced monounsaturated FA content of tissues, blood plasma, milk, and eggs due to inhibition of stearoyl-CoA desaturase-1 (SCD) activity or mRNA expression in tissues (1–7). FA concentrations in plasma lipid fractions of humans were used to calculate Δ5, Δ6, and Δ9 desaturase indices in response to a diet containing a mixture of equal amounts of *cis*-9,*trans*-11-CLA (*c*9,*t*11-CLA) and *t*10,*c*12-CLA (8) or diets containing purified *c*9,*t*11-CLA or *t*10,*c*12-CLA (9). Both reports suggested a reduction in overall capacity for Δ6 and Δ9 desaturation and an increase in Δ5 desaturation capacity due to the CLA mixture or the individual isomers in the diet. Desaturation indices derived from FA concentrations in blood lipid fractions, however, probably were confounded by differential effects of CLA isomers on liver, muscle, adipose tissue, bone, and other tissues. Other confounding factors may include gender (3), metabolic status (1), genetic potential for body fat accretion (1,4), dietary CLA content (6,10), or overall fat content and FA profile of the diet (11,12).

Excess hepatic lipid deposition in response to *t*10,*c*12-CLA may be a consequence of the pronounced inhibition of lipid accumulation and cell development in adipose tissue. In adipocytes, *t*10,*c*12-CLA alters expression of several transcription factors that target genes, including SCD and FA synthase (FAS), required for lipid synthesis and accumulation during cell differentiation and maturation (13). Quantification of cell number and diameter indicated reduced adipocyte cell volume accounted for reduced epididymal fat pad weight of lean rats fed a CLA mixture for 35 d (4). Although subcutaneous adipocyte cell number and diameter in male pigs fed a CLA mixture for 35 d were not altered, the Δ9 desaturation index and SCD activity were reduced (14). Reduced SCD expression in adipose tissue may be linked to enhanced lipid oxidation, as well as reduced triacylglycerol synthesis and storage (15).

An initial evaluation of hepatic SCD response to a dietary CLA mixture or the purified *c*9,*t*11-CLA isomer indirectly indicated that reduced hepatic SCD mRNA expression and reduced hepatic Δ9 desaturation index (ratio of oleic acid to stearic acid) in growing male mice (16) was most likely due to the *t*10,*c*12-CLA in the CLA mixture. A subsequent study with HepG2 cells indicated reduced SCD activity and Δ9 desaturation index (ratio of oleic to stearic) due to *t*10,*c*12-CLA, but SCD mRNA expression was not altered (17). In agreement with the *in vitro* study, hepatic SCD activity, but not SCD mRNA expression, in lactating mice (18) was reduced by *t*10,*c*12-CLA (1% of the diet). In contrast, dietary *t*10,*c*12-CLA (0.5% of the diet) increased the ratio of oleic acid to stearic acid in liver lipids of nonlactating mice, indicating an increase in hepatic SCD activity (19).

Due to these inconsistencies, the present study evaluated carcass composition, FA profiles and total FA in selected tissues, and SCD expression in liver and adipose tissue of growing male

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Abbreviations: CP, crude protein; EE, ether extract; FAS, FA synthase; SCD, stearoyl-CoA-desaturase; *c*9,*t*11-CLA, *cis*-9,*trans*-11-CLA; *t*10,*c*12-CLA, *trans*-10,*cis*-12-CLA.

and female mice fed diets containing 0.0, 0.15, or 0.30% purified *t*10,*c*12-CLA for 6 wk. FA profiles for muscle and bone were used to calculate Δ9 and Δ5 plus Δ6 desaturation indices, which served as estimates of CLA-induced alterations in FA metabolism in lean body mass, for comparison with alterations in desaturation indices in liver. Because a dietary concentration of *t*10,*c*12-CLA as low as 0.1% may be adequate to obtain some of the desirable metabolic responses to this CLA isomer, such as inhibition of tumor metastasis (11), the 0.15% level was included in this study. The 0.30% level approximated the amount of *t*10,*c*12-CLA in a diet containing 0.5–1.0% CLA mixture.

EXPERIMENTAL PROCEDURES

Animals, diets, and sampling. All procedures involving animals were approved by the Virginia Polytechnic Institute and State University Animal Care Committee. Sixty-three male and 63 female, 3- to 4-wk-old CD-1 mice (Harlan, Madison, WI) were housed individually with access to food and water at all times. A 12-h light/12-h dark cycle was maintained throughout the study. For 7 d before the start of the study, all mice were fed Harlan Teklad (Harlan, Madison, WI) Global Rodent Diet (2018) (18% protein, 5% fat) with 3% (wt/wt) high-oleic sunflower oil added. On day 1 of the study, male and female mice were randomly assigned to receive diets containing 3.0% high-oleic sunflower (control), 2.85% high-oleic sunflower oil + 0.15% *t*10,*c*12-CLA, or 2.70% high-oleic sunflower oil + 0.30% *t*10,*c*12-CLA. The free FA form of *t*10,*c*12-CLA (>95% purity) was obtained from Natural Lipids (Hovdebygda, Norway). Mice were fed daily at 1600 h, and food refusals were weighed to estimate intake during the previous 24 h. Samples of each diet were obtained each week and stored at 4°C prior to FA analysis (Table 1).

Body weights were determined twice weekly and the day of sacrifice. At the end of weeks 2, 4, and 6, seven mice per dietary treatment within gender were anesthetized with Metofane® (Pitman-Moore, Inc, Washington Crossing, NJ) prior to cervical dislocation. The liver was removed, rinsed with diethyl pyrocarbonate (Sigma, St. Louis, MO) in distilled water (1:1000, vol/vol), and weighed. A portion of liver was frozen in liquid nitrogen and stored at –80°C prior to mRNA analysis. The remainder of the liver was stored at –20°C prior to FA analysis. Adipose tissue from the inguinal region of females or the epididymal region of males was excised, weighed, frozen in liquid nitrogen, and stored at –80°C prior to mRNA analysis. After evisceration, the head, skin, feet, and tail were removed. Gastrocnemius muscle and femur in the left hind leg of the carcass were excised and stored at –20°C prior to FA analysis. The remainder of the carcass was stored at –20°C prior to drying at 55°C, grinding, and analyses (20) for crude protein (CP) and ether extract (EE) content.

TABLE 1
FA Composition of Diets Fed to Growing Male and Female Mice for 6 Wk

	Dietary *t*10,*c*12-CLA (% total FA)		
FA	0%	0.15%	0.30%
10:0	0.02	0.02	0.02
12:0	0.04	0.05	0.05
14:0	0.12	0.13	0.12
16:0	9.26	9.41	9.18
cis-9-16:1	0.18	0.20	0.19
18:0	2.49	2.65	2.52
trans-11-18:1	0.02	0.02	0.02
cis-9-18:1	34.41	33.09	31.77
18:2n-6	47.34	46.54	46.28
cis-9,*trans*-11-18:2	0.02	0.18	0.32
trans-10,*cis*-12-18:2	0.00	1.37	2.67
18:3n-3	3.72	3.57	3.77
20:0	0.39	0.39	0.38
20:4n-6	0.02	0.02	0.03
Total (μg/mg of diet)	44.07	44.76	46.14

Lipids in diets, liver, femur, and muscle were extracted using the Folch procedure (21). Undecenoic acid (Nu-Check Prep, Inc., Elysian, MN), added to extracted lipids prior to formation of FAME (22), served as an internal standard. Using an Agilent 6890N gas chromatograph equipped with a flame ionization detector (Agilent Technologies, Palo Alto, CA), FAME were separated by a 100 m × 0.25 mm i.d. (0.2 μm film thickness) CP-Sil 88 column (Varian, Lake Forest, CA) protected by a 2.5 m × 0.25 mm i.d. fused silica retention gap. Ultrapure hydrogen at constant pressure was the carrier gas. Pure methyl ester standards (Nu-Check Prep, Inc.) were used for identification and quantification of sample FA (23). Quantities of substrate and product FA (μg/mg of tissue) for selected FA desaturation reactions were used to calculate desaturation indices for liver, muscle, and femur, where desaturation index = substrate/(substrate + product).

Northern blotting. A pkk160 plasmid containing the mouse SCD fragment was donated by Dr. James Ntambi (Department of Biochemistry, University of Wisconsin—Madison), and a pmFAS/CR11 plasmid containing the mouse FAS fragment was obtained from Dr. Hitoshi Shimano (Department of Internal Medicine, University of Tsukuba, Japan). The pDrive cloning vector containing rat β-actin was obtained from Dr. William Huckle (Department of Biomedical Sciences and Pathobiology, Virginia Tech). Plasmids were used to prepare cDNA probes as previously described (18). Total adipose tissue and liver RNA were extracted using TRI REAGENT® (MRC, Cincinnati, OH), separated by electrophoresis on 1% agarose gel containing 0.66 M formaldehyde, and transferred to a Magna Charge nylon membrane (MSI, Westborough, MA) by downward capillary blotting and UV crosslinking. Specific mRNA bands on the membrane were detected using the DIG High Prime DNA Labeling and Detection Starter Kit 2 (Roche Diagnostics Corporation, Indianapolis, IN). Intensity of bands was determined by GelWorks® 1D Intermediate software, version 4.01 (UVP, Inc., Upland, CA). The β-actin band was used for normalization of SCD and FAS mRNA abundance.

Statistical analysis. Data were analyzed as a completely randomized design with a factorial arrangement of treatments using the MIXED procedure of SAS® (Windows version 9.1, Cary, NC). Interactions in the initial model were dietary treat-

ment × gender, treatment × week, gender × week, and treatment × gender × week. Because gender had a significant ($P < 0.05$) effect on most variables in the initial model, data for each gender were analyzed separately. The separate analyses for males and females included a treatment × week interaction. Least-square means ± SEM (combined observations for weeks 2, 4, and 6) for variables within gender are presented in tables and figures. Within each gender, contrasts were performed to test the linear (dose-dependent) effects of the amount of dietary *t*10,*c*12-CLA on each variable.

RESULTS

Table 2 lists overall (mean of weeks 2, 4, and 6) effects of dietary *t*10,*c*12-CLA content on body weight, carcass composition, tissue weights, and total FA in tissues in male and female mice. Dietary *t*10,*c*12-CLA did not alter body weight or food intake (data not shown) during the 6-wk study, regardless of gender, when compared with the control groups. Dry carcass weight, however, was reduced by *t*10,*c*12-CLA. In the initial statistical analysis, which included both genders, a gender by

TABLE 2
Mean[a] Body Weight, Carcass Composition, Tissue Weights, and Tissue FA Content for Male and Female Mice Fed *t*10,*c*12-CLA

	Dietary *t*10,*c*12-CLA				
	0%	0.15%	0.30%	SEM	P <[b]
Body weight (g)					
Male	32.2	32.3	31.8	0.5	0.71
Female	24.7	24.7	25.2	0.3	0.48
Dry carcass					
Weight (g)					
Male	5.88	4.32	4.10	0.20	0.01
Female	3.62	3.16	3.14	0.12	0.01
CP (g)					
Male	2.55	2.42	2.55	0.09	0.51
Female	1.92	1.83	1.91	0.05	0.46
CP (%)					
Male	44.8	56.2	62.2	0.9	0.01
Female	53.6	58.4	61.2	1.0	0.01
EE (g)					
Male	2.10	1.01	0.81	0.12	0.01
Female	1.07	0.79	0.60	0.07	0.01
EE (%)					
Male	35.0	23.7	19.8	1.5	0.01
Female	29.3	24.0	18.7	1.8	0.01
Liver					
Weight (g)					
Male	2.04	2.30	2.36	0.06	0.01
Female	1.50	1.60	1.77	0.05	0.01
Total FA (Ìg/mg)					
Male	38.8	45.0	59.0	3.0	0.01
Female	34.4	43.3	49.7	2.4	0.01
Epididymal adipose (g)	0.73	0.26	0.14	0.04	0.01
Inguinal adipose (g)	0.34	0.20	0.16	0.03	0.01
Muscle					
Weight (g)					
Male	0.19	0.20	0.20	0.006	0.20
Female	0.15	0.14	0.15	0.004	0.20
Total FA (Ìg/mg)					
Male	23.7	14.0	10.7	1.3	0.01
Female	19.7	13.0	11.9	1.2	0.01
Femur					
Weight (mg)					
Male	66.6	73.8	70.0	1.6	0.01
Female	54.6	58.5	58.6	1.5	0.11
Total FA (Ìg/mg)					
Male	4.18	3.47	2.32	0.25	0.01
Female	3.34	2.95	2.65	0.24	0.13

[a]Values are an overall least-square mean for 21 observations, where seven mice within each gender were sacrificed after consuming their assigned diet for 2, 4, or 6 wk.
[b]Probability of a linear effect due to dietary *t*10,*c*12-CLA content.

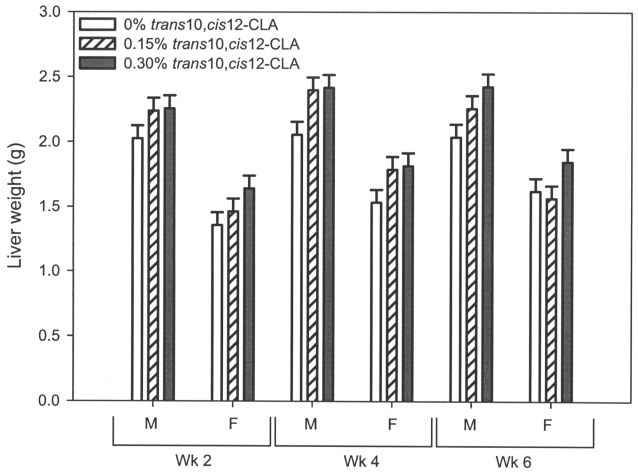

FIG. 1. Biweekly changes in liver weight for male (M) and female (F) mice fed 0.15% or 0.30% *t*10,*c*12-CLA, where $n = 7$. The overall (combined data for weeks 2, 4, and 6) increase in liver weight due to dietary CLA content was linear ($P < 0.01$) for male and female mice, as indicated in Table 2.

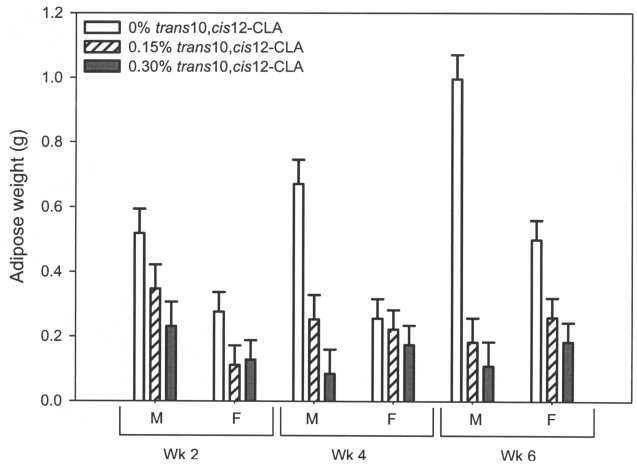

FIG. 2. Biweekly changes in epididymal (M) or inguinal (F) fat pad weight of mice fed 0.15% or 0.30% *t*10,*c*12-CLA, where $n = 7$. The overall (combined data for weeks 2, 4, and 6) reduction in fat pad weight due to dietary CLA content was linear ($P < 0.01$) for male and female mice, as indicated in Table 2.

treatment interaction ($P < 0.05$) resulted due to females responding to the dietary treatments to a lesser extent than males. At 6 wk, carcass weight was 30% lower for males fed 0.15% or 0.30% *t*10,*c*12-CLA, compared with the control, but female dry carcass weight was reduced by only 13%. The reduced carcass weight of males and females was due primarily to a dose-dependent reduction in the amount (g) of EE, whereas amount of carcass CP was similar for all dietary treatments. Thus, CP became a greater percentage of carcass weight as dietary *t*10,*c*12-CLA content increased.

Overall, liver weight of male and female mice increased in response to dietary *t*10,*c*12-CLA in a dose-dependent manner, and the increase was accompanied by an increase in total FA content (Table 2). With the exception of females at 6 wk, liver weight was consistently greater for mice fed *t*10,*c*12-CLA at 2, 4, and 6 wk (Fig. 1). There were overall reductions in inguinal (female) and epididymal (male) fat pad weights in response to increasing dietary *t*10,*c*12-CLA content. The CLA-induced reductions in epididymal adipose tissue weight in males were greater than the reductions in inguinal adipose tissue weight in females due primarily to a biweekly increase in epididymal adipose tissue weight for control-fed male mice compared with a decline in weight for those fed 0.15% or 0.30% *t*10,*c*12-CLA (Fig. 2). Gastrocnemius muscle weight was not altered by dietary treatments, but total FA content of muscle of male and female mice decreased in a dose-dependent manner in response to dietary *t*10,*c*12-CLA. Although femur weight was numerically greater for male and female mice fed 0.15% or 0.30% *t*10,*c*12-CLA, compared with the control groups, the response was significant ($P < 0.01$) only for males. Likewise, the reduction in femur total FA content in response to increasing dietary *t*10,*c*12-CLA content was significant ($P < 0.01$) only for males.

When expressed as a percentage of total FA (data not shown), concentration of *t*10,*c*12-CLA was highest in muscle (0.36% and 0.59% for mice fed 0.15% and 0.30% *t*10,*c*12- CLA, respectively), intermediate in femur (0.20% and 0.39%), lowest in liver (0.15% and 0.31%), and not detectable in these tissues of mice fed the control diet. Palmitic acid (19–24% of total FA), stearic acid (6–10%), oleic acid (29–36%), and linoleic acid (15–21%) were the primary FA in muscle and femur. Palmitic acid (22–23% of total FA) and linoleic acid (17–21%) concentrations in liver were similar to those in muscle and femur. However, stearic acid (17–23%) was greater and oleic acid (20–23%) was lower in liver compared with muscle or femur.

Desaturation indices for liver, gastrocnemius muscle, and femur are listed in Table 3. Potential substrates for the Δ9 desaturation reaction include palmitic acid, stearic acid, and vaccenic acid (*trans*-11-18:1). Desaturation of vaccenic acid was included in Table 3 because it accounted for a small portion of total FA in all diets (Table 1). The desaturation indices for 18:0 and *trans*-11-18:1 in liver of males and females were reduced by dietary *t*10,*c*12-CLA. In contrast, the desaturation indices for 16:0 and 18:0 in muscle (males and females) and femur (males only) were elevated by *t*10,*c*12-CLA. The desaturation index for 18:2n-6, which includes Δ5 and Δ6 desaturases, in liver was not influenced by dietary *t*10,*c*12-CLA content, but it was reduced in muscle (males and females) and femur (males only) of mice fed *t*10,*c*12-CLA. The 18:3n-3 desaturation index in liver of male mice was elevated in response to dietary *t*10,*c*12- CLA, but was not altered in muscle or femur of either gender.

FA analysis of adipose tissue was not possible, because all available tissue was needed for RNA isolation. Evaluation of adipose tissue RNA revealed linear reductions in SCD (Fig. 3) and FAS (Fig. 4) mRNA, regardless of gender, due to increasing dietary *t*10,*c*12-CLA content. In addition, SCD and FAS expression in adipose tissue of females was greater ($P < 0.05$) than in males. Unlike adipose tissue, overall hepatic SCD mRNA expression was not affected by dietary *t*10,*c*12-CLA content or gender (data not shown).

TABLE 3
Desaturation Indices[a] for Liver, Gastrocnemius Muscle, and Femur of Male and Female Mice Fed *t*10,*c*12-CLA

	Dietary *t*10,*c*12-CLA				
	0%	0.15%	0.30%	SEM	P <[b]
Liver					
16:0 to 16:1					
Male	0.97	0.97	0.97	0.003	0.58
Female	0.99	0.98	0.98	0.001	0.13
18:0 to 18:1					
Male	0.49	0.45	0.37	0.018	0.01
Female	0.55	0.51	0.48	0.017	0.02
trans-11-18:1 to *c*9,*t*11-CLA					
Male	0.89	0.75	0.66	0.040	0.01
Female	0.84	0.67	0.59	0.048	0.01
18:2n-6 to 20:4n-6					
Male	0.74	0.76	0.79	0.017	0.16
Female	0.75	0.77	0.77	0.019	0.85
18:3n-3 to 20:5n-3					
Male	0.83	0.87	0.90	0.016	0.01
Female	0.80	0.83	0.86	0.019	0.14
Muscle					
16:0 to 16:1					
Male	0.80	0.87	0.90	0.006	0.01
Female	0.85	0.89	0.91	0.008	0.01
18:0 to 18:1					
Male	0.13	0.17	0.21	0.010	0.01
Female	0.15	0.17	0.22	0.014	0.01
trans-11-18:1 to *c*9,*t*11-CLA					
Male	0.45	0.52	0.53	0.034	0.22
Female	0.43	0.38	0.38	0.031	0.33
18:2n-6 to 20:4n-6					
Male	0.90	0.83	0.80	0.013	0.01
Female	0.87	0.84	0.79	0.010	0.01
18:3n-3 to 20:5n-3					
Male	0.90	0.89	0.86	0.017	0.29
Female	0.86	0.80	0.83	0.020	0.07
Femur					
16:0 to 16:1					
Male	0.79	0.81	0.86	0.011	0.01
Female	0.84	0.85	0.86	0.011	0.52
18:0 to 18:1					
Male	0.18	0.20	0.26	0.013	0.01
Female	0.22	0.23	0.25	0.014	0.44
trans-11-18:1 to *c*9,*t*11-CLA					
Male	0.38	0.40	0.51	0.042	0.06
Female	0.41	0.35	0.40	0.038	0.40
18:2n-6 to 20:4n-6					
Male	0.85	0.83	0.77	0.013	0.01
Female	0.82	0.82	0.82	0.013	0.94
18:3n-3 to 20:5n-3					
Male	0.81	0.80	0.79	0.008	0.31
Female	0.80	0.78	0.79	0.010	0.55

[a]Desaturation index = substrate/(substrate + product). Values are an overall least-square mean for 21 observations, where seven mice within each gender were sacrificed after consuming their assigned diet for 2, 4, or 6 wk.
[b]Probability of a linear effect due to dietary *t*10,*c*12-CLA content.

DISCUSSION

Despite reductions in weight of the dry carcass and carcass fat content in this study, total body weight and food intake were not influenced by 0.15% or 0.30% dietary *t*10,*c*12-CLA. Previous dose-response studies using male mice indicated diets containing 0.5% or more CLA mixture were needed for reductions of body weight or energy intake (10,24), and these reductions eventually were attributed to dietary *t*10,*c*12-CLA rather than *c*9,*t*11-CLA (25).

A dry carcass without head, skin, feet, and tail was used in the present study to obtain estimates of changes in lipid and protein content of structural body components in response to dietary *t*10,*c*12-CLA. Carcass weight, carcass lipid content,

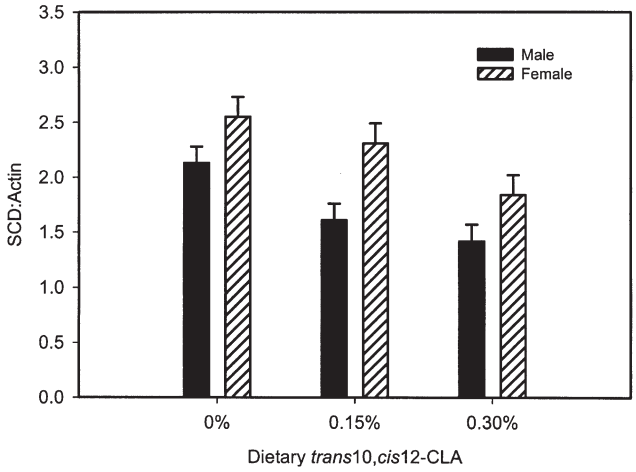

FIG. 3. SCD mRNA in epididymal adipose tissue of male mice and inguinal adipose tissue of female mice fed 0.15% or 0.30% *t*10,*c*12-CLA. Each bar represents the mean for 21 mice, where seven mice were sacrificed at 2, 4, or 6 wk within the 6-wk study. The decline in SCD mRNA due to dietary *t*10,*c*12-CLA content was linear ($P < 0.05$) for male and female mice, and SCD mRNA abundance was greater ($P < 0.05$) for females compared with males.

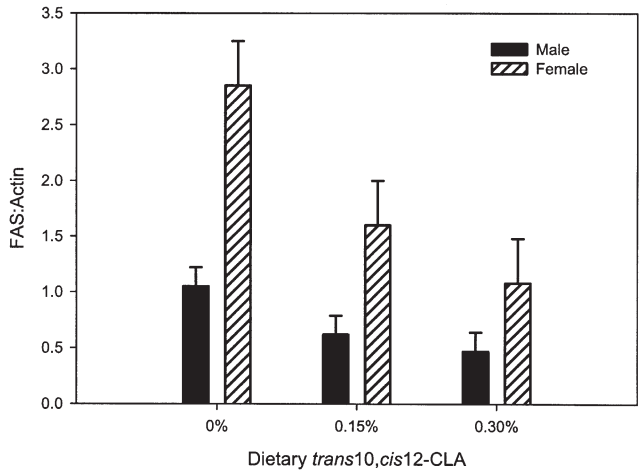

FIG. 4. FAS mRNA in adipose tissue of male and female mice fed *t*10,*c*12-CLA. Each bar represents the mean for 21 mice, where seven mice were sacrificed at 2, 4, or 6 wk within the 6-wk study. The decline in FAS mRNA due to dietary *t*10,*c*12-CLA content was linear ($P < 0.05$) for male and female mice, and FAS mRNA abundance was greater ($P < 0.05$) for females compared with males.

and weight of the epididymal or inguinal fat pad were reduced in a dose-dependent manner. Reduction in carcass fat content accounted for a majority of the reduction in dry carcass weight, because protein content of the carcass was not altered. Although there was a dose-dependent decrease in total FA in muscle of both genders, femur FA content was reduced to a greater extent in males (45%) than in females (21%). In association with reduced deposition of lipids in peripheral tissues, however, was a 15–18% increase in weight of the liver. Liver enlargement was reported for male mice fed a diet containing 1.0% CLA mixture (40% *t*10,*c*12-CLA) (24), male mice fed 1.5% CLA mixture (48% *t*10,*c*12-CLA) (26), female mice fed 1% CLA mixture (36% *t*10,*c*12-CLA) (27), female mice fed 0.4% purified *t*10,*c*12-CLA (28), and male and female mice fed 1.0% purified *t*10,*c*12-CLA (29). Hepatic enlargement was associated with a 3- to 7-fold increase in triacylglycerol content (26,28). The dose-dependent increase in liver weight and total FA content noted in this study suggested the lower threshold for these hepatic responses may be 0.15% *t*10,*c*12-CLA or less in a diet.

Gastrocnemius muscle weight of male and female mice was not affected by dietary treatments. However, femur weight increased due to 0.15% and 0.30% dietary *t*10,*c*12-CLA. Increased bone weight may be due to a decrease in 20:4n-6 concentration in bone (30), which also was observed in the current study (data not shown). Inadequate 20:4n-6 may reduce production of eicosanoids, including prostaglandin E2 (1), essential for bone resorption and osteoclastic activity (31). In response to reduced osteoclastic activity, net bone turnover may be low and lead to increased bone forming rate.

Previous studies evaluated potential causes of reduced body fat deposition, including adipocyte apoptosis and insulin resistance, due to CLA mixtures or purified *t*10,*c*12-CLA (13,27,28,29). Female mice fed a diet with 1.0% CLA mixture for 5 mon had reduced FAS mRNA expression in white adipose tissue (27). In contrast, SCD mRNA expression was reduced in mouse 3T3-L1 pre-adipocytes in response to *t*10,*c*12-CLA (13). In the current study, there was a linear decrease in expression of FAS and SCD mRNA in response to dietary *t*10,*c*12-CLA in male and female mice. Males, however, had lower FAS and SCD mRNA in adipose tissue than females. Lower expression of FAS and SCD in males suggested that their adipose tissue depots may be more susceptible to the inhibitory effects of *t*10,*c*12-CLA, which appeared to be confirmed by the greater dose-dependent reduction in epididymal fat pad weight of males compared with inguinal fat pad weight reduction of females at 4 and 6 wk (Fig. 2).

FA desaturation indices as indicators of Δ9, Δ5, and Δ6 desaturation in this study were based on the concentration (μg/mg) of a selected FA in a tissue and calculated as a ratio of substrate to (substrate plus product). In the liver and most other tissues, SCD catalyzes the conversion of 16:0 to *cis*-9-16:1 and 18:0 to *cis*-9-18:1 by insertion of a double bond at the Δ9 position (32). Likewise, SCD desaturates *trans*-11-18:1 to *c*9,*t*11-18:2 (33). Highly unsaturated FA, such as 20:4n-6 and 20:5n-3, are formed from 18:2n-6 or 18:3n-3 by Δ5 and Δ6 desaturation. Thus, an increase in a desaturation index (Table 3) suggested a reduction in activity of a corresponding desaturase enzyme. Increases in 16:0 and 18:0 desaturation indices for muscle (male and female) and femur (males only) were observed for mice fed *t*10,*c*12-CLA, indicating a corresponding reduction in SCD activity as previously reported for mouse mammary tissue (18) and pre-adipocytes (13). In contrast, reductions in 18:0 and *trans*-11-18:1 desaturation indices for liver of male and female mice were observed in the present study, and suggested enhanced hepatic SCD activity. Expression of SCD mRNA in the liver of male and female mice in the present study, however, was not altered by dietary *t*10,*c*12-

CLA. Hepatic SCD mRNA in lactating mice also was not altered by dietary *t*10,*c*12-CLA, but SCD activity was reduced (18). The *t*10,*c*12 CLA isomer most likely altered hepatic SCD activity by post-translational mechanisms rather than gene transcription (17).

The hepatic 18:2n-6 desaturation index was not altered by dietary treatments in this study, but the index was reduced in muscle (males and females) and femur (males only). The proportion of 20:4n-6, the product of 18:2n-6 desaturation and elongation, in bone and muscle of male and female mice was increased (data not shown). An increase in activity of Δ5 or Δ6 desaturase in muscle and femur may be a metabolic response to compensate for the apparent decrease in Δ9 desaturase activity noted above. In contrast, dietary *t*10,*c*12-CLA caused an increase in the hepatic 18:3n-3 desaturation index of male mice, suggesting inhibition of Δ5 or Δ6 desaturation. There was a reduction in Δ6 desaturation in Hep2G cells in response to *t*10,*c*12-CLA when the ratio 20:3n-6 to18:2n-6 was used as an index (34). Likewise, the ratio of (18:3n-6 + 20:3n-6) to 18:2n-6 in plasma phospholipids suggested potential inhibition of Δ6 desaturation in response to daily consumption of purified *t*10,*c*12-CLA by overweight men and women (9). In addition, hepatic Δ6 desaturase activity was reduced by *c*9,*t*11-CLA and *t*10,*c*12-CLA added to microsomal incubations (35). Thus, alterations in 18:2n-6 or 18:3n-3 desaturation indices in this study may have resulted primarily from changes in activity of Δ6 desaturases.

Our results indicated gender accounted for minor differences in the capacity of adipose tissue, via FAS and SCD, to respond to the inhibitory actions of dietary *t*10,*c*12-CLA on lipid synthesis and storage. However, results also indicated that evaluations of lower concentrations of dietary *t*10,*c*12-CLA are needed to establish the appropriate amount of this CLA isomer required for acceptable long-term reductions in body fat content with minimal negative effects on liver lipid content and essential FA desaturation in muscle, bone, and other tissues. For example, conjugated EPA (36) apparently has fewer of the undesirable side effects associated with *t*10,*c*12-CLA. Thus, both conjugated isomers could be compared at similar dietary concentrations in future studies to determine their desirable and undesirable dose-dependent characteristics in male and female animals.

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Biocatalysis of Linoleic Acid to Conjugated Linoleic Acid

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ABSTRACT: CLA refers to a group of geometrical and positional isomers of linoleic acid (LA) with conjugated double bonds. CLA has been reported to have diverse health benefits and biological properties. Traditional organic synthesis is highly capital-intensive and results in an isomeric mixture of CLA isomers. Biotechnology presents new alternatives to traditional lipid manufacturing methods. The objective of this study was to examine the effect of protein isolation procedures on linoleate isomerase (LAI) recovery from microbial cells and biocatalysis of LA to CLA. Protein isolation experiments were carried out using *Lactobacillus acidophilus* L1 and two strains of *Lactobacillus reuteri* (ATCC 23272 and ATCC 55739). Under the same assay conditions, ATCC 55739 had the highest LAI activity among the microbial cultures examined in this study. Efficiency of cell lysis methods, which included various combinations of lysozyme and mutanolysin treatments in combination with sonication and osmotic rupture of cells with liquid nitrogen, was very low. Although treatment of cell material with a detergent (octylthioglucoyranoside) freed a significant amount of LAI activity into the solution, it was not sufficient to recover all the LAI activity from the residual cells. Crude LAI preparations produced mainly the *cis*-9,*trans*-11 CLA isomer. Time and substrate/protein ratio had a significant effect on biocatalysis of LA to CLA. It appears that the mechanism and kinetics of enzymatic conversion of LA to CLA are quite complex and requires further research using pure LAI preparations.

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CLA refers to a group of geometrical and positional isomers of linoleic acid (LA) with conjugated double bonds. Although a total of 54 CLA isomers are possible (1), only about 20 CLA have been identified, including *cis*,*cis*; *trans*,*trans*; *cis*,*trans*; and *trans*,*cis* isomers of 7,9; 8,10; 9,11; 10,12; and 11,13 C18 diene acids (2). It is believed that the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers are the most bioactive CLA forms (3). CLA has been reported to have diverse health benefits and biological properties including being anticarcinogenic (4,5), antiatherogenic (6,7), antidiabetic (8), antiobesity (9), antioxidative (10), immunomodulative (11), antibacterial (2), cholesterol lowering, and growth promoting (12). It also has been reported that dietary intake of CLA helps to build lean muscle, reduce deposition of fat, and mitigate some undesirable aspects of the immune response such as anorexia and protein breakdown (13,14). Most of these effects have been demonstrated only in animal studies.

CLA can be produced through organic synthesis, microbial fermentation, enzymatic isomerization, or genetic engineering/bioengineering (15). Traditional organic synthesis is highly capital-intensive and results in an isomeric mixture of CLA. There have been several reports that microorganisms containing the enzyme linoleic acid isomerase (LAI) are capable of converting LA to CLA (14,16–19). Hence, CLA production by fermentation and utilization of microorganisms containing LAI may be a viable alternative to organic synthesis. It is also possible that pure enzymes containing isomerase activity can be used to manufacture CLA. A great advantage of biocatalysis by whole microorganisms or purified enzymes is that there is high specificity for the isomers that are produced.

A rumen bacterium, *Butyrivibrio fibrisolven*, was the first identified to convert LA to CLA (20). Since then there have been numerous reports that a large number of benign bacteria, which are naturally present in the environment and intestinal tract of ruminant animals, can convert LA to CLA (14,17–19). Fairbank *et al.* (21) were the first to detect CLA in *Lactobacillus* cultures. Later Pariza and Yang (14) developed a method for production of *cis*-9,*trans*-11 CLA utilizing *Lactobacillus* sp. Their patent covers the conversion of LA to CLA by using whole bacterial cells of *L. reuteri* PYR8. Jiang *et al.* (19) screened 19 different strains of lactobacilli, lactococci, streptococci, and propionibacteria for their ability to produce CLA. Two strains of *Propionibacterium freudenreichii* ssp. *freudenreichii* and one strain of *P. freudenreichii* ssp. *sheramnii* were able to convert free LA to CLA, and *cis*-9,*trans*-11 was the dominant isomer. Alonso *et al.* (18) tested four different cultures, two strains each of *L. acidophilus* and *L. casei* for their ability to produce CLA from free LA. All the cultures were able to produce free CLA in MRS broth supplemented with LA.

Although conversion of LA to CLA by microorganisms has been reported extensively, literature on the utilization of enzyme extracts and purified enzyme preparations for LA isomerization is limited (22). A cell-free protein preparation catalyzing the isomerization of LA and linolenic acids was obtained from *B. fibrisolvens* (23). It was reported that occasionally cultures of intact cells of *B. fibrisolvens* tended to settle rapidly and failed to isomerize LA readily when incubated with the substrate. This phenomenon was explained by the production of a capsular material, which altered the permeability of the cell to LA. However, isomerase isolated from these cells had the

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Abbreviations: LA, linoleic acid; LAI, linoleate isomerase; PAI, polyenoic FA isomerase.

ability to rapidly convert LA to CLA. Park *et al.* (24) isolated LAI from *B. fibrisolvens* A-38 cultured anaerobically. They also determined the molecular weight and partial amino acid sequence of the enzyme. The isomerase was a single polypeptide with a molecular weight of 19 kDa. Yang (25) isolated and characterized a LAI enzyme from *L. reuteri*. The linoleic acid isomerase of *L. reuteri* MRS8 was membrane-bound, and had a molecular weight of more than 100 kDa. Optimal activity of the enzyme was in the pH range of 4.7 to 5.4. Recently, Lin *et al.* (26) prepared enzyme extracts from *L. acidophilus* and *P. freudenreichii* ssp. *shermanii.* These extracts were used for isomerization of LA, and *trans*-10,*cis*-12; *cis*-11,*tran*-13; and *cis*-9,*trans*-11 were the three major CLA isomers produced. Liavonchanka *et al.* (22) reported overproduction of a polyenoic FA isomerase (PAI) in *Escherichia coli* and its purification as a yellow-colored protein. PAI catalyzes the double-bond isomerization of LA to CLA. A world patent also describes determination of linoleate isomerase enzyme nucleic acid and amino acid sequences and genetic modification of the naturally occurring enzyme (27).

Although limited data is available on the conversion of LA to CLA using microbial enzymes, the efficiency of enzyme recovery from whole bacterial cells during protein isolation has not been reported. Hence, the objective of this study was to examine the effect of protein isolation procedures on efficiency of enzyme recovery from whole microbial cells. This study also examines the effect of substrate/protein ratio and time on CLA isomer formation using solubilized crude protein solutions.

MATERIALS AND METHODS

Culture growth. Culture growth experiments (1, 2, and 4 L) were carried out using *L. acidophilus* L1 (obtained from the stock culture collection of the Food Microbiology Laboratory at the Food and Agricultural Products Research and Technology Center, Oklahoma State University), and two strains of *L. reuteri* (ATCC 23272 and ATCC 55739) (commercial cultures). All the cultures studied in this project were subcultured three times (1% inoculum into 10 mL MRS broth, incubated 18 h at 37°C) just prior to use. Then a 1% inoculum was added into the final volume (1, 2, and 4 L) of MRS broth (Difco Laboratories, Detroit, MI) and incubated at 37°C for 18 h.

Protein isolation. Several enzyme isolation protocols were tested for highest activity in the crude preparations. A typical crude enzyme preparation protocol included the following steps. (1) Cells were harvested from the growth medium by centrifugation at 10,000*g* for 10 min at 4°C and washing the pellet with 0.85% NaCl (30 mL) and buffer (either phosphate buffer pH 7 or tris-maleate buffer pH 5.4). (2) Cell disruption: Three different methods were tested. The first involved lysozyme treatment (7 mg/mL, Sigma, St. Louis, MO) (20°C for 10 min) and sonication for 10 min (Model 450 Sonifier, Branson Ultrasonics Corporation, Danbury, CT) in an ice-bath. The second was lysozyme treatment (1 hr at 37°C) then addition of 4 M NaCl and sonication for 10 min in an ice-bath. Sonication was carried out in 2-min intervals to avoid overheating of the solution. The third was treatment with an enzyme mixture containing 1 mM EDTA, 24% sucrose, 2% lysozyme, and 50 U/mL mutanolysin (Sigma, St. Louis, MO) at 37°C for 90 min and osmotic rupture of the cells (cells were frozen in liquid nitrogen and thawed in warm water; this procedure was repeated twice). The degree of cell lysis was examined with a stain microscope after each treatment. (3) After the cell lysis, the solution (lysate) was centrifuged at 10,000*g*, 4°C for 60 min, to separate solid and liquid fractions. All the protein fractions obtained at each step of the isolation process were tested for enzyme activity using free LA as a substrate. (4) The solid fraction that had the enzyme activity was treated with a detergent, octylthioglucoyranoside (OSGP) (Sigma, St. Louis, MO), at 4°C for 2 h to solubilize LAI from the membrane fraction. The detergent solution and a solid fraction suspension in buffer were mixed at 1:1 to obtain the final detergent concentrations of 0.1, 0.3, 0.5, 1, and 2%. (5) Detergent solubilized protein mixture was ultrafiltered through 500, 300, and 100 kDa pore size membranes.

Protein assay. Protein amount in the samples was determined by the Bradford method (28) using a standard curve of BSA (Sigma, St. Louis, MO) in 6–200 μg protein range. Coomassie Brillant Blue G-250 (Sigma, St. Louis, MO) at 0.033 g/L concentration was used for protein-dye binding. The absorbance of the solutions was determined at 595 nm by using a UV spectrophotometer (Model Beckman DU640, Beckman Coulter, Inc., Fullerton, CA). Coomassie brillant blue without protein solution was used as blank.

Enzyme assay. Enzyme activity tests were carried out with 100 μL of 50 mg LA/mL methanol, and extracts containing 100–600 μg of protein. The volume of the substrate protein mixture was adjusted to 3 mL by adding tris maleate (pH = 5.4) or phosphate buffer (pH 7). Then the mixture was incubated at 37°C for 3 h in a mechanical shaker (1024 Tecator, at speed setting 5, Foss North America, Eden Prairie, MN).

Extraction and methylation procedure. After internal standard addition (0.25 mg/mL heptadecanoic acid in methanol), lipids in enzyme assay solution were extracted three times with 5 mL of hexane (total 15 mL). The hexane extract was dried first over anhydrous sodium sulfate (ACS grade, EMD Chemicals Inc., Gibbstown, NJ) followed by nitrogen drying using a Reacti-Vap evaporation unit (Model 18780, Pierce, Rockford, IL) set at 40°C. Then 100 μL of 1.0 N NaOH in methanol and 1 mL of BF_3-methanol solutions (10–15% solution, Sigma, St. Louis, MO) were added to the dried lipid sample. Methylation was carried out at room temperature for 30 min. Three milliliters of saturated aqueous NaCl and 2 mL of hexane were added to the methylation solution, which was mixed well. The hexane layer was recovered and dried with Na_2SO_4. Before injection into the GC, the hexane phase was concentrated to 1 mL.

Methyl esters of CLA were quantified by using a HP 6890 Series GC system equipped with a flame ionization detector (Agilent Technologies, Palo Alto, CA). A fused silica capillary column SP-2560 (100 m × 0.25 mm × 0.2 μm film thickness) from Supelco (Bellefonte, CA) was used for the analysis. The oven temperature was programmed as follows: temperature was maintained at 135°C for 1 min, increased from 135°C to 165°C with 1°C/min heating rate, maintained at 165°C for 1

min, increased from 165°C to 167°C with 0.5°C/min rate, maintained at 167°C for 1 min, increased from 167°C to 240°C at 1.75°C/min, and maintained at 240°C for 5 min. Helium was used as carrier gas at a flow rate of 1.0 mL/min. The inlet temperature was 250°C. The split ratio was 1:50. One microliter of sample was injected into the GC by an autosampler (HP 7683, HP Company, Wilmington, DE). Isomers of CLA in the samples were identified by comparing retention times of standard CLA isomers (*cis*-9,*trans*-11; *trans*-10,*cis*-12; *cis*-9,*cis*-11; and *trans*-9,*trans*-11) and the mass spectra with those of the authentic compounds. MS (5973 Network Mass Selective Detector, Agilent Technologies, Palo Alto, CA) parameters were as follows: MS transfer line 280°C, ion source 230°C, and MS quadrupole temperature 150°C. The ionization energy was 70 eV. The scan range and rate were 100–600 amu and 2 scans/s, respectively. The peaks were also confirmed with NIST/EPA/NIH Mass Spectral Library (Version 2.0).

RESULTS AND DISCUSSION

A number of large-scale (1, 2, and 4 L) culture growth experiments were carried out using *L. acidophilus* L1 and two strains of *L. reuteri* (ATCC 23272 and ATCC 55739). Microbial cells were harvested when the culture reached the stationary growth phase. Wet cell yield was about 3–4 g/L growth medium. All the microbial cultures examined in this study produced similar cell yields. Several cell lysis protocols were tested for their efficiency to release enzyme activity from cell material. Figure 1 shows the effect of lysozyme and lysozyme + sonication treatment on cell integrity. A significant portion of the cells were still intact even after treatment of whole cells with lysozyme (20°C for 10 min) followed by 10 min sonication. The same trend was observed for all of the cultures examined in this study, indicating that cell disruption would be a major challenge for large and/or commercial isolation of LAI from these strains. Enzyme activity assay and the GC method used in this study do not cause isomerization of LA in the absence of LAI (Fig. 2). Base-catalyzed BF_3 methylation at room temperature appears to be an effective method for methylation with no isomerization of conjugated dienes (29).

Preliminary enzyme assays carried out using protein solutions (100 μg protein for all assays) obtained from lysozyme treatment (20°C for 10 min) and sonication (10 min) of microbial cells indicated differences in enzyme activity in the crude protein solutions (Fig. 3). *Lactobacillus reuteri* ATCC 55739 gave the highest LAI activity in crude enzyme preparations resulting in 10% (w/w) LA conversion to CLA in 3 h. Protein solutions obtained from two other cultures examined in this study had lower enzyme activity than ATCC 55739 (<1% LA conversion to CLA) under the same conditions. Main CLA isomers formed by ATCC 55739 were *cis*-9,*trans*-11 and *trans*-10,*cis*-12, which are believed to be the most biologically active CLA isomers. Higher LAI activity in ATCC 55739 was also confirmed by comparing enzyme assays performed with three whole cell cultures tested in this study. Hence, further protein purification experiments were carried out using ATCC 55739. An increase in lysozyme concentration, treatment temperature, and time did not improve LA1 activity in crude enzyme preparations.

A series of protein isolation experiments were performed to examine the effect of isolation procedures on LAI activity and conversion of LA to CLA (Fig. 4). Treatment of whole ATCC 55739 cells (S1) with CelLytic (Sigma, St. Louis, MO), which is proprietary blend of a detergent and enzymes optimized for in-culture bacterial cell lysis did not release detectable amount of LAI activity into solution. Nonetheless, solubilization and removal of some of the proteins that do not posses LAI activity from the cell material during CelLytic treatment caused concentration of LAI activity (mg CLA/mg cell) in the residual cell material (Fig. 4, S2). Further treatment of residual cell material with a lysozyme/mutanolysin mixture in tris maleate buffer at pH 5.4 followed by osmotic cell rupture resulted in release of some LAI activity into solution (L3) and reduction in enzyme activity in

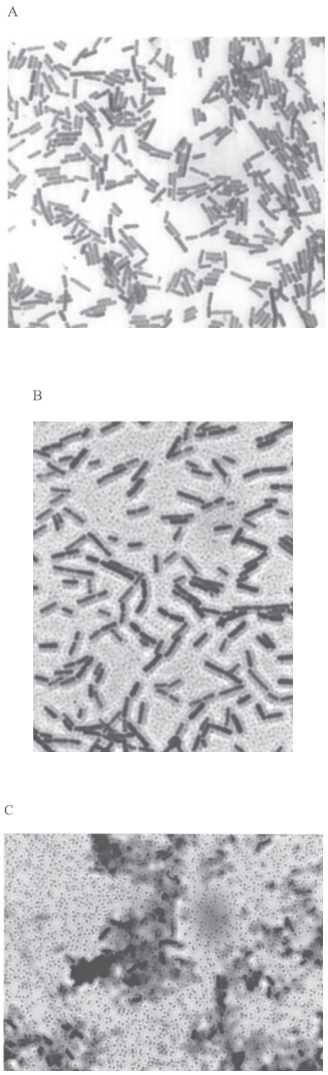

FIG. 1. Effect of lysozyme treatment and sonication on *Lactobacillus reuteri* (ATCC 55739) cell integrity. A: Intact cells before treatment; B: Lysozyme treatment at 20°C for 10 min—majority of the cells are still intact; C: Lysozyme treatment at 20°C for 10 min + 10 min sonication—although there is some cell disruption, significant number of cells are still intact.

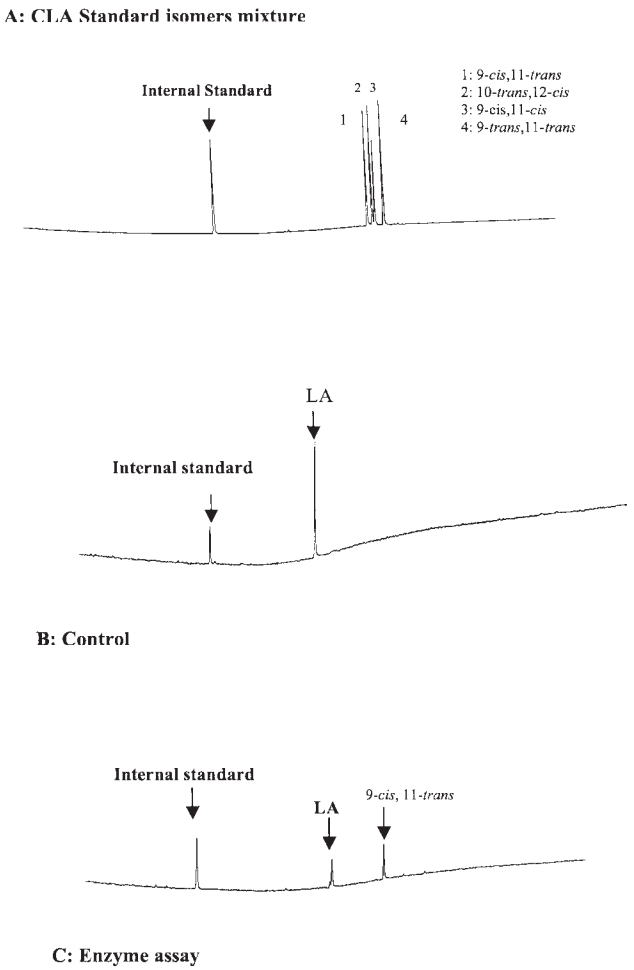

FIG. 2. Effect of fatty acid methylation method and enzyme assay on isomerization of LA.

the residual cell material (S3) (Fig. 4). However, a significant amount of LAI activity still remained in the residual cell material (S3), indicating that LAI may be cell-bound. Although treatment of residual cells with a detergent solution (octylthioglucopyranoside) released some more enzyme activity into the solution (L4), it was not possible to recover all the LAI activity from cell material (S4). Even after a second detergent treatment, a significant amount of LAI activity was detected both in solution (L5) and in the cell residue (S5). Increasing the detergent amount and incubation time for one treatment was not as effective as two sequential detergent treatments in LAI recovery from the cell residue. Ultrafiltration of combined protein solutions (L3 + L4 + L5) through a 100-kDa membrane facilitated concentration of LAI activity in the retentate (L6). These results indicate that expression of LAI in a microorganism that would secrete this enzyme into a growth medium is essential for economical feasibility of large-scale LAI isolation/production.

Effects of time and substrate/protein ratio on CLA isomer formation were also examined. Minor revisions to protein isolation protocols described in the Material and Methods section were made to maximize enzyme recovery and obtain enough protein solution to run these tests. The revised protein isolation protocol was as follows.

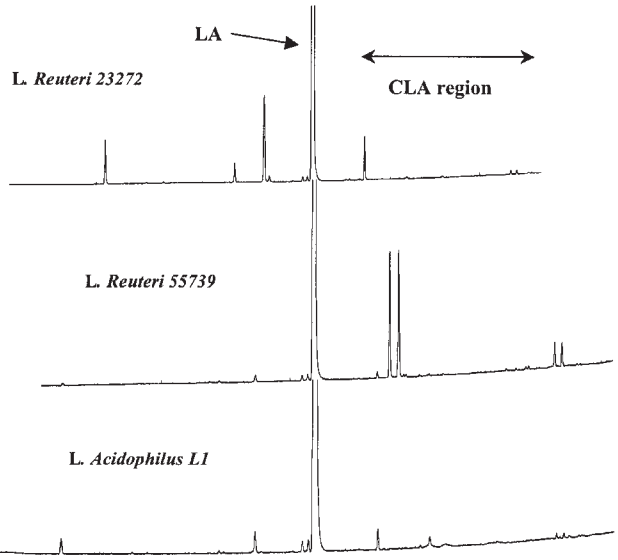

FIG. 3. Effect of microbial culture type on enzymatic conversion of LA to CLA. Enzyme assays were carried out using substrate/protein ratio of 0.2 (w/w) for 3 h. Linoleic acid in free form and protein solutions obtained after lysozyme treatment (20°C, 10 min) + sonication were used for the assay.

(1) Whole cells were treated with an enzyme mixture (phosphate buffer pH 7) followed by centrifugation at 8,000*g* for 20 min to separate solid (SA) and liquid phases (LiqA). (2) Osmotic

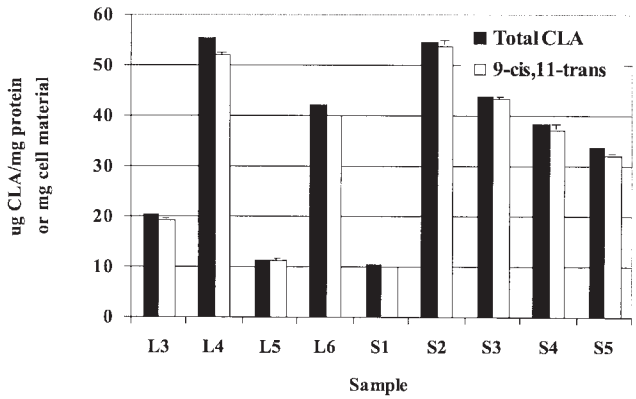

FIG. 4. Effect of protein isolation procedures on LAI recovery and CLA formation. ATCC 55739 culture was used for these experiments. **S1**: Whole ATCC 55739 cells; **S2**: Residual cell material after treatment of S1 with 10 mL CelLytic B cell lysis reagent (Sigma St. Louis, MO) at 20°C for 15 min; **S3**: Residual cell material after treatment of S2 with a lysozyme + mutanolysin mixture (Materials and Methods, Protein Isolation Section, 2-c); **S4**: Residual cell material after treatment of S3 with a detergent solution (Materials and Methods, Protein Isolation Section, 4 at 0.3% detergent concentration in the final solution); **S5**: Residual cell material after treatment of S4 with a detergent solution (Materials and Methods, Protein Isolation Section, 4 at 0.3% detergent concentration in the final solution); **L3**: Protein solution obtained after treatment of S2 with a lysozyme + mutanolysin mixture (Materials and Methods, Protein Isolation Section, 2-c); **L4**: Protein solution obtained after treatment of S3 with a detergent solution (Materials and Methods, Protein Isolation Section, 4 at 0.3% detergent concentration in the final solution); **L5**: Protein solution obtained after treatment of S4 with a detergent solution (Materials and Methods, Protein Isolation Section, 4 at 0.3% detergent concentration in the final solution); **L6**: Retentate obtained after ultrafiltration of combined L3 + L4 + L5 solution through a 100 kDa membrane. Protein content of the final solution was 0.96 mg/mL.

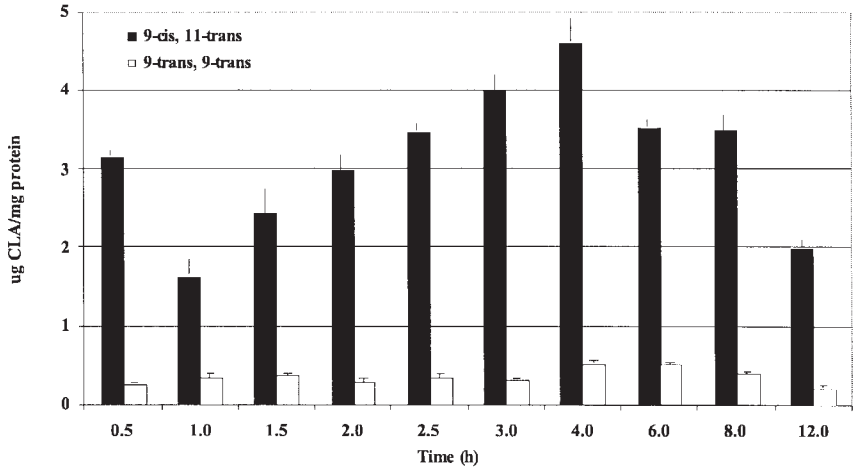

FIG. 5. Effect of time on enzymatic conversion of LA to *trans*-9,*trans*-11 CLA isomer. Crude enzyme preparations were obtained from ATCC 55739 cultures as explained in the text.

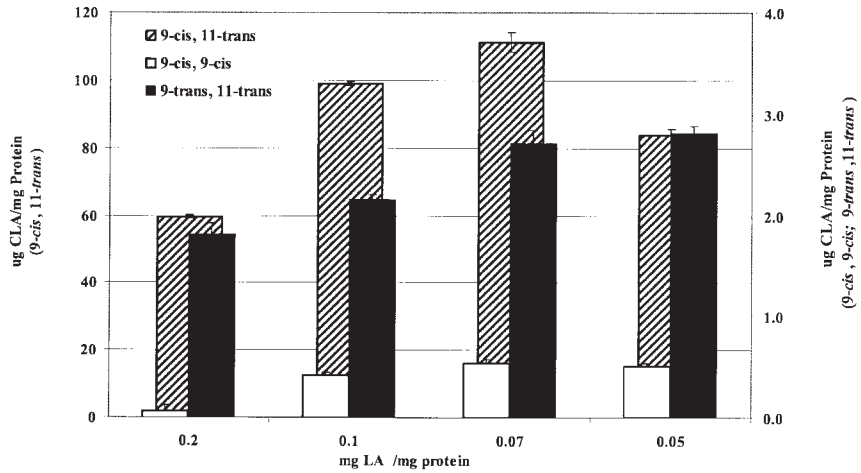

FIG. 6. Effect of substrate/protein ratio on enzymatic conversion of LA to *trans*-9,*trans*-11 CLA isomer. Crude enzyme preparations were obtained from ATCC 55739 cultures as explained in the text.

rupture of the SA was followed by centrifugation at 8,000*g* for 20 min to separate solid (SB) and liquid phases (LiqB). (3) Treatment of SB with a detergent solution (0.12 g/50 mL membrane fraction + 50 mL detergent solution) was followed by centrifugation at 8,000*g* for 20 min at 4ºC to separate solid (SC) and liquid phases (LiqC).

The detergent treatment was repeated twice. Then protein solutions containing LAI activity (LiqA + LiqB + LiqC) were combined and ultrafiltered through a 300-kDa membrane. Retentate fraction from the ultrafiltration was used for the evaluation. Although there was some enzyme activity in the permeate fraction, the majority of the LAI activity was retained in the retentate fraction which had a concentration of 0.96 mg protein/mL solution.

Effect of time on CLA isomer production is shown in Figure 5. Maximum LA conversion to CLA was observed at 4 h (Table 1). Both *cis*-9,*trans*-11 and *trans*-9,*trans*-11 isomer formations were maximum at this time. Incubation of LA with protein solution longer than 4 h did not improve *cis*-9,*trans*-11 and *trans*-9,*trans*-9 isomer accumulation in the assay solution. Rather, a decrease in LA conversion to CLA was observed after 4 h (Fig. 5). These results were in agreement with the LA tests carried out in the assay solutions, which showed a slight increase in LA concentration at 4 h and after.

TABLE 1
Conversion of LA to CLA as a Function of Time[a]

Time (h)	0.5	1	1.5	2	2.5	3	4	6	8	12
LA converted (%, w/w)	3.4	2.0	2.8	3.3	3.8	4.3	5.1	4.0	3.9	2.2

[a]See Figure 6 for experimental conditions.

Substrate/enzyme ratio is an important parameter for understanding the mechanism of biocatalysis of LA to CLA. Effect of substrate/enzyme ratio on CLA formation was examined using the solutions obtained from the same protein isolation procedure described above. Decreasing LA/protein ratio (wt/wt) from 0.2 to 0.07 improved LA to CLA conversion (Fig. 6). The same trend was observed for all three CLA isomers detected in the assay solutions, *cis*-9,*trans*-11; *cis*-9,*cis*-9, and *trans*-9,*trans*-11. However, a further decrease in LA/protein ratio from 0.07 to 0.05 caused a significant decrease in biocatalysis of LA to CLA.

This study clearly showed that incomplete cell lysis is a major impediment for efficient isolation of LAI from microbial cells. Evaluation of effect of time and substrate/enzyme ratio on CLA formation indicates that the mechanism and kinetics of biocatalysis of LA to CLA are quite complex. Utilization of crude LAI preparations further complicates the evaluation. A better understanding of the mechanism of enzymatic CLA production requires further research using higher purity LAI preparations. Expression of LAI activity in microbial cells which would secrete the enzyme into a growth medium would be the key for the success of future research and development work in the field.

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Syntheses of Conjugated Octadecadienoic Acids

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ABSTRACT: Three approaches for the synthesis of octadecadienoic acids with conjugated double bond systems are presented: synthesis of (10*Z*,12*Z*)-octadecadienoic acid *via* an enyne-substructure; the use of an educt with a conjugated double bond system for the synthesis of (10*E*,12*E*)-octadecadienoic acid; and the Suzuki cross coupling for the synthesis of (7*E*,9*Z*)-octadecadienoic acid.

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There are a number of positional and geometrical isomers of octadecadienoic acids, showing conjugated double bonds, that are referred to as conjugated linoleic acid (CLA). They are found predominantly in milk and meat, with 9*Z*,11*E*-CLA as the main isomer. In ruminants, CLA are formed by *Butyrivibrio fibrisolvens* in the rumen and also in adipose tissue during dehydrogenation of monounsaturated FA. Since their discovery, their potential benefits to human health have become evident. In various animal bioassays, CLA showed anticarcinogenic, antiatherogenic, and antidiabetogenic properties and proved to be a modulator for body composition. These studies were mostly conducted using CLA mixtures prepared by base-catalyzed isomerization of linoleic acid or with pure 9*Z*,11*E*-CLA or 10*E*,12*Z*-CLA, the only isomers that are commercially available. There is evidence that the 10*E*,12*Z*-isomer reduces the uptake of lipids by the adipocytes, whereas the 9*Z*,11*E*-isomer is active in inhibiting carcinogenesis in animal models, and both have been suggested to inhibit atheriosclerosis (1,2). Because of these differences in activities it is important to use pure stereoisomers in further investigations dealing with the biological significance of these compounds.

Several syntheses of pure isomers have been reported. Methyl 9*Z*,11*E*-CLA was prepared from methyl ricinoleate in 83% purity (3). Higher purities could be obtained by alkali isomerization of methyl linoleate with low-temperature crystallization (4). Pure 9*Z*,11*E*- and 9*Z*, 11*Z*-isomers were also synthesized by zinc reduction of methyl santalbate (5). A mixture of the 9*Z*,11*E*-isomer and the 9*E*,11*E*-isomer, labeled with deuterium, was prepared and separated by a combination of reversed-phase and silver silica chromatography in purities of >97–99% (6). Lipase-induced esterification of a mixture of stereoisomeric acids yielded butyl(9*Z*,11*E*)-octadecadienoate in purities of 90% (7). A mixture of 9*Z*,11*E*- and 10*E*,12*Z*-CLA was prepared in technical grade by selective isomerization of linoleic acid with metal catalysts (8). Pure 9*Z*,11*E*-, 10*E*,12*Z*-, and 10*Z*,12*Z*-CLA were synthesized by using stereochemically defined 1,2-dichloroethene as a building block (9). All these approaches yielded single isomers on purification of mixtures of two or more isomers. Purification, either by crystallization or chemoenzymatic methods, always resulted in laborious multistep procedures causing significant loss of material. Here we report new and easy stereoselective methods for the synthesis of (10*E*,12*E*)-octadecadienoic acid using an educt with a conjugated double bond system, (10*Z*,12*Z*)-octadecadienoic acid *via* an enyne-substructure, and the Suzuki cross coupling for the synthesis of (7*E*,9*Z*)-octadecadienoic acid.

MATERIALS AND METHODS

Mass spectra were obtained with the instruments GC 8000 Series/MD800 (Fisons instruments) using a fused-silica capillary column (CP 8944, VF-5ms factor four WCOT; 30 m × 0.25 mm i.d., 0.25 μm film thickness; Varian, Darmstadt, Germany). GC-FTIR data were obtained with the instruments Finnigan Trace GC Ultra GC (Thermo Electron, Dreieich, Germany) with a Vertex 70 spectrometer (Bruker, Rheinstetten, Germany) using a fused-silica capillary column (CP-Sil 88, 100 m × 0.25 mm i.d., 0.2 μm film thickness). NMR spectra were recorded in $CDCl_3$ with a Bruker 400 MHz spectrometer. Liquid chromatography was performed with 60 Å silica gel from MP Biomedicals (Illkirch, France).

All reactions of air- and water-sensitive materials were performed in dried glassware under an argon atmosphere. Most chemicals were purchased from Aldrich; 1-heptyne, dry THF, and dry pyridine were purchased from Fluka (Buchs, Switzerland), while $PdCl_2[PhCN]_2$ was obtained from Merck (Darmstadt, Germany). Dichloromethane was dried over calcium hydride and distilled.

*Preparation of 8-bromo-1-octanol (**2**).* 1,8-Octanediol (**1**) (9.4 g, 64 mmol), 50 mL aqueous hydrobromic acid (48%), and toluene were placed in a liquid-liquid extractor. The extractor was kept at 80°C for 40 h, and the toluene in the flask was heated under reflux. Owing to the circulation of the solvent, the 8-bromo-1-octanol formed during the reaction was transferred to the flask. The toluene containing the reaction product from the flask was washed once with water, twice with saturated

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Abbreviations: DHP, dihydropyrane; DiBAlH, diisobutylaluminumhydride; DMAP, dimethylaminopyridine; DMF, dimethylformamide; $HB(cy)_2$, dicyclohexylborane; OPG, oxygen + protecting group; PDC, pyridiniumdichromate; PPTS, 4-pyridiniumtoluenesulfonic acid; *p*TsOH, *p*-toluenesulfonic acid.

sodium hydrogen carbonate solution, and then with brine. The organic layer was dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was used directly for the next step of the synthesis. Yield: 12.6 g (94%).

*Preparation of 1-(2′-tetrahydropyranyloxy)-8-bromooctane (**3**).* Crude 8-bromo-1-octanol **(2)** (12.6 g) and 1.5 g (7.8 mmol) PPTS in 120 mL of dry dichloromethane were added dropwise at 0°C to a solution of 6 mL (55 g, 66 mmol) of 3,4-dihydropyran in 30 mL of dry dichloromethane. The mixture was stirred overnight at 4°C and poured into ice water. The phases were separated, and the aqueous phase was extracted three times with diethyl ether. The combined organic layers were washed with diluted sodium hydrogen carbonate solution and with brine, then dried over anhydrous magnesium sulfate; then the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 10:1 hexane/ethyl acetate. Yield: 14.8 g (79 %). MS [*m/z* (%)]: 111 (8), 101 (9), 85 (100), 69 (4), 67 (12), 55 (43), 43 (33), 41 (30), 39 (23). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 1.29–1.48 (*m*, 8H, 3-H to 6-H), 1.49–1.64 (*m*, 6H)/1.67–1.76 (*m*, 1H)/1.79–1.90 (*m*, 3H) (3′-H to 5′-H, 2-H and 7-H), 3.37–3.44 (*m*, 3H, 1-H_a and 8-H), 3.48–3.55 (*m*, 1H, 6′-H_a), 3.69–3.77 (*m*, 1H, 1-H_b), 3.84–3.91 (*m*, 1H, 6′-H_b), 4.56–4.60 (*m*, 1H, 2′-H).

*Preparation of (2E,4E)-decadienol (**5**).* Freshly distilled *(2E,4E)*-decadienal **(4)** (16 g, 10 mmol), dissolved in 65 mL dry dichloromethane, was added to 21 mL (21 mmol) diisobutylaluminum hydride (1 M in dichloromethane) at –78°C over a period of 15 min. The mixture was stirred for an additional 4.5 h at –78°C, and 0.65 mL methanol as well as 20 mL saturated Seignette salt (potassium sodium tartrate) solution was added. After warming to room temperature, the mixture was stirred for an additional 40 min and extracted twice with diethyl ether. The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. Yield of the crude product: 1.6 g (79%). MS [*m/z* (%)]: 154 (M^+, 10), 136 (8), 110 (9), 97 (12), 84 (42), 83 (74), 79 (46), 70 (32), 67 (59), 67 (59), 57 (19), 55 (80), 43 (28), 41 (100), 39 (34). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.88 (*t*, 3H, *J* = 6.8 Hz, 10-H), 1.22–1.42 (*m*, 6H, 7-H to 9-H), 2.03–2.11 (*m*, 2H, 6-H), 4.11 (*d*, 2H, *J* = 6.1 Hz, 1-H), 5.65–5.73 (*m*, 2H, 2-H and 5-H), 5.99–6.08 (*m*, 1H)/6.15–6.24 (*m*, 1H) (3-H and 4-H).

*Preparation of (2E,4E)-decadienylacetate (**6**).* Catalytic amounts of dimethylaminopyridine and 1.6 g of the crude *(2E,4E)*-decadienol **(5)** were dissolved in 60 mL dry pyridine. To this mixture was added dropwise 1 mL (11 mmol) acetic anhydride, and stirring was continued overnight. After pouring it into 50 mL of ice water, the mixture was extracted three times with hexane. The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 10:1 hexane/ethyl acetate. Yield: 1.8 g (67%). MS [*m/z* (%)]: 196 (M^+, 6), 153 (2), 136 (2), 110 (4), 97 (2), 80 (16), 79 (26), 70 (3), 67 (15), 55 (10), 53 (4), 43 (100), 41 (20), 39 (9). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.79 (*t*, 3H, *J* = 6.8 Hz, 10-H), 1.14–1.34 (*m*, 6H, 7-H to 9-H), 1.95–2.00 (*m*, 5H, 6-H and 2′-H), 4.47 (*d*, 2H, *J* = 6,6 Hz, 1-H), 5.53 (*dt*, 1H, *J* = 15.3 Hz/6.6 Hz)/5.65 (*dt*, 1H, *J* = 15.3 Hz/7.1 Hz)/(2-H and 5-H), 5.88–5.98 (*m*, 1H)/6.16 (*dd*, 1H, *J* = 15.3 Hz/4.8 Hz) (3-H and 4-H).

*Preparation of (10E,12E)-1-(2′-tetrahydropyranyloxy)-octadecadiene (**7**).* In a flask, 0.170 g (7 mmol) magnesium turnings were covered with dry THF, and 2 drops of 1,2-dibromoethane were added. Subsequently, a solution of 1.35 g (4.6 mmol) 1-(2′-tetrahydropyranyloxy)-8-bromooctane **(3)** in 10 mL dry THF was added in such a manner that the solution remained gently boiling. After complete addition, the mixture was heated under reflux for an additional 30 min.

At room temperature, 0.40 g (2 mmol) (2*E*,4*E*)-decadienylacetate **(6)** and 0.8 mL lithium tetrachlorocuprate (0.1 M in THF) in 15 mL dry THF were stirred for 15 min. At –20°C, the solution of 1-(2′-tetrahydropyranyloxy)-8-octylmagnesiumbromide was added, and the mixture was stirred at 0°C for 4 h. After warming to room temperature, the mixture was stirred for an additional hour and poured into an ammonium chloride/hexane mixture at 0°C, which was then extracted three times with hexane. The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 30:1 hexane/ethyl acetate. Yield: 0.41 g (60%). MS [*m/z* (%)]: 350 (M^+, 0.2), 266 (1), 149 (1), 137 (1), 135 (2), 121 (3), 109 (3), 101 (7), 95 (11), 85 (100), 79 (17), 69 (7), 67 (32), 57 (12), 55 (27), 43 (22), 41 (70), 39 (11). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.85 (*t*, 3H, *J* = 6.9 Hz, 18-C), 1.17–1.37 (*m*, 16H, 2-H to 7-H and 16-H to 17-H), 1.45–1.60 (*m*, 8H)/1.65–1.73 (*m*, 1H)/1.75–1.85 (*m*, 1H) (3′-H to 5′-H and 8-H and 15-H), 1.96–2.07 (*m*, 4H, 9-H, and 14-H), 3.36 (*dt*, 1H, *J* = 9.4 Hz/6.7 Hz, 1-H_a), 3.43–3.51 (*m*, 1H, 6′′-H_a), 3.7 (*dt*, 1H, *J* = 9.7 Hz/7.1 Hz 1-H_b), 3.81–3.88 (*m*, 1H, 6′-H_b), 4.53–4.57 (*m*, 1H, 2′H), 5.46–5.58 (*m*, 2H, 10-H, and 13-H), 5.90–6.01 (*m*, 2H, 11-H, and 13-H),

*Preparation of (10E,12E)-octadecadienol (**8**).* Catalytic amounts of 4-toluenesulfonic acid and 0.41 g (1,2 mmol) (10*E*,12*E*)-1-(2′-tetrahydropyranyloxy)-octadecadiene **(7)** were dissolved in 10 mL methanol. After stirring for 30 min at 60°C, solid sodium hydrogen carbonate was added, and the suspension was stirred for an additional 15 min. *In vacuo* the mixture was reduced to *ca*. 30% of the original volume. After the addition of water, the mixture was extracted three times with diethyl ether. The combined organic layers were dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. Yield of the crude product: 0.37 g (*ca*. 100%). MS [*m/z* (%)]: 266 (M^+, 4), 150 (1), 135 (3), 110 (5), 95 (23), 81 (33), 79 (37), 69 (11), 67 (56), 55 (42), 43 (27), 41 (100), 39 (21). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.88 (*t*, 3H, *J* = 6.8 Hz, 18-H), 1.24–1.41 (*m*, 16H, 4-H to 8-H and 15-H to 17-H), 1.51–1.60 (*m*, 2H, 2-H), 2.00–2.08 (*m*, 4H, 9-H, and 14-H), 3.63 (*t*, 2H, *J* = 6,6 Hz, 2-H), 5.51–5.60 (*m*, 2H, 10-H, and 13-H), 5.94–6.04 (*m*, 2H, 11-H and 12-H).

*Preparation of (10E,12E)-octadecadienoic acid (**A**).* Crude (10*E*,12*E*)-octadecadienol **(8)** (0.37 g) was dissolved in 5 mL acetone, and Jones reagent [acidic solution of Cr(VI)] was

added dropwise at 0°C, until the reddish-brown color did not switch to green. After addition of one drop of 2-propanol, the suspension was filtered, and the mixture was reduced *in vacuo* to *ca*. 25% of the original volume. After addition of 10 mL water and 10 mL diethyl ether, the aqueous layer was extracted four times with diethyl ether. The combined organic layers were extracted four times with a 1 N solution of sodium hydroxide. Subsequently, the alkaline soapy solution was acidified with 2 N hydrochloric acid to pH 4 and was extracted four times with diethyl ether. The combined organic layers were dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was recrystallized from methanol to give 10 mg (3%) of the desired product. For NMR data see Table 1.

*Preparation of (Z)-1-bromo-1-heptene (**11**).* 1-Heptyne **(9)** (1.7 g, 17.7 mmol) and 2.1 g 1,3,2-benzodioxaborole (17.7 mmol) were placed in a dry 25-mL flask. The mixture was heated for 2 h at 70°C and cooled to room temperature. After addition of 5 mL dry dichloromethane, the solution was cooled to –20°C; and 2 mL bromine, dissolved in 8 mL dry dichloromethane, were added dropwise. After the addition was completed, 20 mL 2 N sodium hydroxide solution was added carefully at –80°C, and the solution was stirred for 1 h at room temperature. The solution was then extracted three times with dichloromethane; the combined organic layers were washed with brine and dried over anhydrous magnesium sulfate. The solvent was removed *in vacuo,* and the product was purified by kugelrohr distillation to give 2.3 g (72%) of (*Z*)-1-bromo-1-heptene. MS [*m/z* (%)]: 178 (M^+, 8), 176 (M^+, 8), 136 (1), 134 (1), 121 (7), 119 (7), 97 (19), 70 (31), 55 (100), 41 (62). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.89 (*t*, 3H, *J* = 6.9 Hz, 7-H), 1.24–1.46 (*m*, 6H, 4-H to 6-H), 2.16–2.22 (*m*, 2H, 3-H), 6.05–6.15 (*m*, 2H, 1-H, and 2-H).

*Preparation of 1-(2′-tetrahydropyranyloxy-)-10-undecyne (**13**).* At 0°C a solution of 2.8 g (3 mL, 33 mmol) 2,3-dihydropyrane in 15 mL dry dichloromethane was added dropwise to a solution of 5.0 g 10-undecyn-1-ol **(12)** (30 mmol) and 0.75 g pyridinium-4-toluene sulfonate (3 mmol) in 60 mL dry dichloromethane. The mixture was stirred overnight at 4°C, poured into ice water, and extracted three times with diethyl ether. The combined organic layers were washed with diluted sodium hydrogen carbonate and brine and dried over anhydrous magnesium sulfate. The solvent was removed *in vacuo,* and the crude product was purified by flash chromatography on silica using 8:1 hexane/ethyl acetate. Yield: 5.6 g (74%). MS [*m/z* (%)]: 251 (M^+, 1), 101 (17), 95 (11), 85 (100), 67 (23), 55 (25), 41 (26). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 1.23–1.43 (*m*, 12H, 2-H to 7-H), 1.47–1.63 (*m*, 6H)/1.66–1.75 (*m*, 1H)/1.77–1.87 (*m*, 1H) (3′-H to 5′-H and 4-H), 1.93 (*t*, 1H, *J* = 2.6 Hz, 1-H), 2.17 (*dt*, 2H, *J* = 2.5/7.1 Hz, 3-H), 3.38 (*dt*, 1H, *J* = 9.4/6.7 Hz, 6′-Ha), 3.46–3.53 (*m*, 1H, 1-Ha), 3.72 (*dt*, 1H, *J* = 9.4/6.7 Hz, 6′-Hb), 3.83–3.90 (*m*, 1H, 1-Hb)

*Preparation of (Z)-1-(2′-tetrahydropyranyloxy)-octadec-10-yn-12-ene (**14**).* (*Z*)-1-Bromo-1-heptene **(11)** (0.71 g, 4 mmol), 76 mg copper(I) iodide (0.4 mmol), 76 mg bis(benzonitrile)-dichloropalladium(II) ($PdCl_2[PhCN]_2$, 0.2 mmol), and 12 mL piperidine were placed in a 25-mL dry flask. To this suspension 2.0 g (8 mmol) 1-(2′-tetrahydropyranyloxy-)-10-undecyne (**13**) was added at room temperature. After stirring for 2 h, saturated aqueous ammonium chloride solution was added to the suspension, which was extracted three times with diethyl ether. The combined organic layers were washed with 0.2 N hydrochloric acid, saturated sodium hydrogen carbonate solution, and twice with water. The organic layer was dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo.* The crude product was purified by flash chromatography over silica using 50:1 hexane/ethyl acetate. Yield: 1.2 g (86%). MS [*m/z* (%)]: 348 (M^+, 2), 264 (1), 219 (2), 205 (1), 177 (1), 163 (1), 121 (4), 101 (5), 85 (100), 79 (21), 55 (32), 41 (60). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.89 (*t*, 3H, *J* = 6.9 Hz, 18-H), 1.26–1.45 (*m*, 16H, 2-H to 7-H and 16-H to 17-H), 1.50–1.62 (*m*, 8H)/1.67–1.75 (*m*, 1H)/1.79–1.88 (*m*, 1H) (3′-H to 5′-H and 8-H and 15-H), 2.24–2.35 (*m*, 4H, 9-H and 14-H), 3.38 (*dt*, 1H, *J* = 9.7 Hz/6.6 Hz, 1-H_a), 3.47–3.53 (*m*, 1H, 6′-H_a), 3.73 (*dt*, 1H, *J* = 9.66 Hz/6.9 Hz, 1-H_b), 3.83–3.90 (*m*, 1H, 6′-H_b), 4.53–4.59 (*m*, 1H, 2′-H), 5.40–5.45 (*m*, 1H, 12-H), 5.80 (*dt*, 1H, *J* = 10.7 Hz/7.4 Hz, 13-H).

*Preparation of (10Z,12Z)-1-(2′-tetrahydropyranyloxy)-octadecadiene (**15**).* Dropwise and at 0°C, 0.43 mL (9 mmol) borane dimethyl sulfide complex was added to a solution of 0.90

TABLE 1
^{1}H NMR Data of Synthesized Octadecadienoic Acids

	^{1}H NMR (400 MHz, $CDCl_3$): d (ppm)
10*E*,12*E* **(A)**	0.88 (*t*, 3H, *J* = 7.1 Hz, 18-H), 1.24–1.41 (*m*, 16H, 4-H bis 8-H and 15-H bis 17-H), 1.59–1.67 (*m*, 2H, 3-H), 2.01–2.07 (*m*, 4H, 9-H and 14-H), 2.34 (*t*, 2H, *J* = 7.6 Hz, 2-H), 5.51–5.61 (*m*, 2H, 10-H and 13-H), 5.95–6.03 (*m*, 2H, 11-H and 12-H)
10*Z*,12*Z* **(B)**	0.89 (*m*, 3H, 18-H), 1.24–1.37 (*m*, 16H, 4-H to 8-H and 15-H to 17-H), 1.47–1.55 (*m*, 2H, 3-H), 1.60–1.67 (*m*, 4H, 9-H and 14-H), 2.31–2.37 (*m*, 2H, 2-H), 6.09–6.16 (*m*, 2H, 10-H and 13-H), 6.81–6.88 (*m*, 2H, 11-H and 12-H)
7*E*,9*Z* **(C)**	0.88 (*t*, 3H, *J* = 6.9 Hz, 18-H), 1.22–1.45 (*m*, 16H, 4-H, 5-H and 12-H bis 17-H), 1.5–1.69 (*m*, 2H, 3-H), 2.07–2.18 (*m*, 4H, 6-H and 11-H), 2.35 (*t*, 2H, *J* = 7.5 Hz, 2-H), 5.31 (*dt*, 1H, *J* = 10.9/7.6 Hz, 10-H), 5.63 (*dt*, 1H, *J* = 7.2/15.1 Hz, 7-H), 5.93 (*dd*, 1H, *J* = 10.8 Hz, 9-H), 6.29 (*ddd*, 1H, *J* = 15.1/11.0/1.2 Hz, 8-H)

mL (9 mmol) cyclohexene in 21 mL dry hexane. The mixture was stirred until a white precipitate had formed (*ca*. 10 min).

At 0°C, 3.6 mL of the freshly prepared dicyclohexylborane solution was added to 0.35 g (*Z*)-1-(2′-tetrahydropyranyloxy-)-octadec-10-yn-12-ene **(14)** (1 mmol), dissolved in 20 mL dry hexane. The mixture was stirred for 2 h at room temperature and diluted with 8 mL dry THF. After the addition of 0.50 mL acetic acid, the mixture was heated to 50°C for 3 h. The solution was cooled to room temperature and hydrolyzed with 2 mL sodium hydroxide solution (5 M) and 0.44 mL of 30% aqueous hydrogen peroxide solution. The mixture was stirred for an additional 30 min, poured into ice water, and extracted three times with hexane. The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 100:1 hexane/ethyl acetate. Yield: 216 mg (62%). MS [*m/z* (%)]: 350 (M^+, 0.1), 266 (1), 101 (12), 95 (10), 85 (100), 81 (13), 67 (25), 55 (16), 41 (24). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.89 (*t*, 3H, *J* = 6.9 Hz, 18-C), 1.25–1.40 (*m*, 16H, 2-H to 7-H and 16-H to 17-H), 1.48–1.61 (*m*, 8H)/1.67–1.75 (*m*, 1H)/ 1.80–1.86 (*m*, 1H) (3′-H to 5′-H and 8-H and 15-H), 2.13–2.20 (*m*, 4H, 9-H and 14-H), 3.38 (*dt*, 1H, *J* = 9.7 Hz/6.6 Hz, 1-H_a), 3.46–3.53 (*m*, 1H, 6′-H_a), 3.73 (*dt*, 1H, *J* = 9.7 Hz/6.9 Hz, 1-H_b), 3.84–3.91 (*m*, 1H, 6′-H_b), 4.56–4.59 (*m*, 1H, 2′H), 5.40–5.48 (*m*, 2H, 10-H and 13-H), 6.20–6.30 (*m*, 2H, 11-H and 12-H).

*Preparation of (10Z,12Z)-octadecadienol (**16**).* (10*Z*,12*Z*)-1-(2′-Tetrahydropyranyloxy)-octadecadiene **(15)** (0.216 g, 0.62 mmol) was dissolved in 10 mL methanol, and catalytic amounts of *p*-toluenesulfonic acid were added. After the mixture had been stirred overnight at room temperature, solid sodium hydrogen carbonate was added, and the suspension was stirred for an additional 15 min. *In vacuo* the mixture was reduced to *ca*. 25% of the original volume, water was added, and the solution was extracted three times with diethyl ether. The combined organic layers were dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 5:1 hexane/ethyl acetate. Yield: 0.188 g (*ca*. 100%). MS [*m/z* (%)]: 266 (M^+, 4), 248 (1), 149 (2), 135 (7), 121 (11), 109 (14), 95(40), 81 (65), 67 (100), 55 (47), 41 (73). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.89 (*t*, 3H, *J* = 6.9 Hz, 18-C), 1.25–1.40 (*m*, 20H, 2-H to 8-H and 15-H to 17-H), 1.51–1.60 (*m*, 4H, 9-H and 14-H), 3.63 (*t*, 2H, *J* = 6,6 Hz, 1-H), 5.40–5.49 (*m*, 2H, 10-H and 13-H), 6.20–6.28 (*m*, 2H, 11-H and 12-H).

*Preparation of (10Z,12Z)-octadecadienoic acid (**B**).* (10*Z*,12*Z*)-Octadecadienol (**16**) (97 mg, 0.4 mmol), dissolved in 5 mL acetone, was added dropwise to Jones reagent at 0°C until the reddish-brown color did not switch to green. After addition of one drop of 2-propanol, the suspension was filtered. Subsequently, the mixture was concentrated *in vacuo* to *ca*. 25% of the original volume. Then, after addition of 10 mL water and 10 mL diethyl ether, the aqueous layer was extracted four times with diethyl ether. The combined organic layers were dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 2:3 hexane/ethyl acetate. Yield: 56 mg (50%). For NMR data see Table 1.

*Preparation of (Z)-1-bromo-1-decene (**19**).* In a dry flask 4.2 g (40 mmol) 1-decyne **(17)** and 4.8 g (40 mmol) 1,3,2-benzodioxaborole were heated for 2 h at 70°C. The mixture was cooled to room temperature and dissolved in 25 mL dry dichloromethane. At –20°C, 4.1 mL bromine dissolved in 18 mL dry dichloromethane was added, and the solution was brought to –80°C. Sodium hydroxide solution, 41 mL, 2 N, was added slowly so that the temperature inside the flask did not rise above –20°C. After stirring for 1 h at room temperature, the mixture was extracted three times with dichloromethane, washed with brine, dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was used in the next step without further purification.

*Preparation of 7-octyne-1-ol (**21**).* Dry 1,3-diaminopropane (110 mL) and 15 g (214 mmol) lithium were stirred until all lithium had been dissolved (30 min). The obtained blue solution was kept at 70°C until a white suspension formed, and the blue color disappeared (overnight). The mixture was cooled to room temperature, and 16 g (142 mmol) potassium *tert*-butanolate was added. The resulting yellow solution was stirred for an additional 20 min. The mixture was cooled in a water bath, and 4.5 g (36 mmol) 2-octyne-1-ol (**20**) was slowly added so that the temperature remained below 30°C. After stirring for 30 min at room temperature, the solution was poured into ice water and extracted four times with hexane. The combined organic layers were washed with water, 10% hydrochloric acid, brine, dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 2:1 hexane/ethyl acetate. Yield: 2.5 g (55%). MS [*m/z* (%)]: 107 (2), 95 (13), 93 (30), 79 (60), 67 (64), 55 (36), 41 (100). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 1.30–1.44 (*m*, 4H, 3-H and 4-H), 1.46–1.58 (*m*, 4H, 2-H and 5-H), 1.91 (*t*, 1H, *J* = 2.7 Hz, 8-H), 2.15 (*dt*, 2H, *J* = 2.5 Hz/7.0 Hz, 6-H), 3.59 (*t*, 2H, *J* = 6.6 Hz, 1-H).

*Preparation of (E)-1-octenylboronic acid (**22**).* 1,3,2-Benzodioxaborole (21 mL, 19 mmol) was slowly added to 1.2 g (9.5 mmol) 7-octyn-1-ol (**21**) dissolved in 5 mL dry THF. After the formation of hydrogen had ceased, the mixture was refluxed for 5 h. After cooling to room temperature, 10 mL water was added, and stirring was continued for 2 h. The generated white precipitate was put in the refrigerator overnight and was isolated upon filtration at 0°C the next day. The precipitate was washed with ice-cold water to remove excess catechol. The crystals were dried over P_2O_5. The crude product was used in the next step without further purification. Yield: 0.81 g (35%).

*Preparation of (7E,9Z)-octadecadien-1-ol (**23**).* A mixture of 0.72 g (3 mmol) (*E*)-1-octenylboronic acid (**22**), 0.41 g (6 mmol) sodium ethanolate in 3 mL ethanol (2 M), and catalytic amounts of 2(3)-*tert*-butyl-4-methoxyphenol (BHA) were dissolved in 3 mL toluene. The mixture was added dropwise at room temperature to a mixture of 0.46 g (2 mmol) (*Z*)-1-bromo-1-decene (**19**) and 0.10 g (0.1 mmol) $Pd(PPh_3)_4$, dissolved in 6 mL toluene. The solution was heated for 2 h at

65°C. After cooling to room temperature, 6 mL 30% aqueous hydrogen peroxide and 6 mL 2 N sodium hydroxide solution were added slowly to destroy the excess borane. The reaction mixture was extracted three times with diethyl ether, and the combined organic layers were washed with water, brine, dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 5:1 hexane/ethyl acetate. Yield: 0.34 g (61%). MS [*m/z* (%)]: 266 (M^+, 11), 248 (2), 149 (3), 135 (7), 121 (11), 109 (11), 95 (25), 79 (70), 67 (100), 55 (36), 41 (83). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.88 (*t*, 3H, *J* = 6.9 Hz, 18-H), 1.24–1.44 (*m*, 18H, 3-H to 5-H and 12-H to 17-H), 1.54–1.61 (*m*, 2H, 2-H), 2.07–2.17 (*m*, 4H, 6-H and 11-H), 3.64 (*t*, 2H, *J* = 6.6 Hz, 1-H), 5.31 (*dt*, 1H, *J* = 10.7 Hz/7.4 Hz, 10-H), 5.64 (*dt*, 1H, *J* = 15.0 Hz/7.1 Hz, 7-H), 5.93 (*dd*, 1H, *J* = 10.9 Hz/10.9 Hz, 9-H), 6.30 (*ddd*, 1H, *J* = 10.9 Hz/15.0 Hz/1.28 Hz, 8-H).

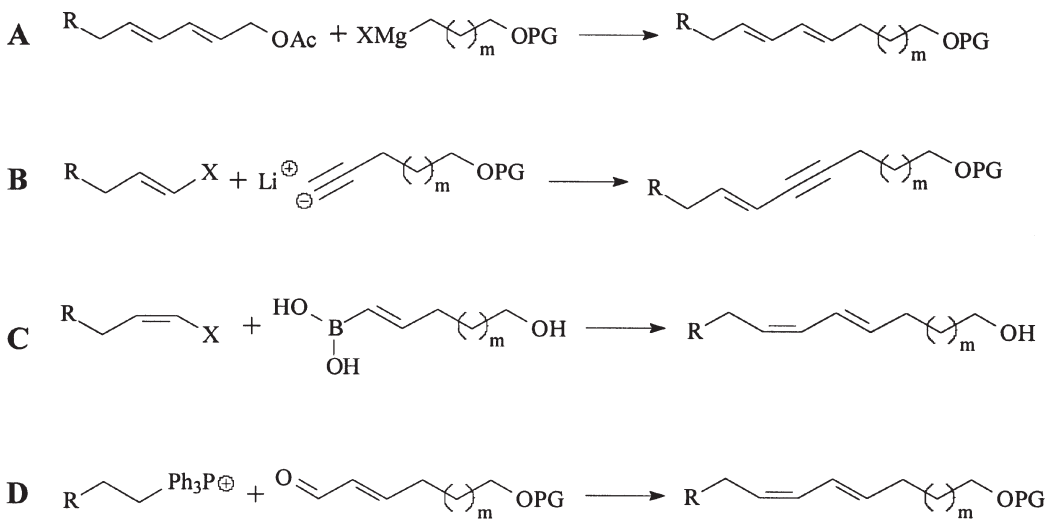
*Preparation of (7E,9Z)-octadeca-7,9-dienoic acid (**C**).* At room temperature and for 2.5 h 0.34 g (1.3 mmol) (7*E*,9*Z*)-octadecadien-1-ol (**23**), 1.95 g (5.2 mmol) pyridinium dichromate, and 7.8 mL dimethylformamide (DMF) were stirred. The mixture was poured into water, which was acidified with hydrochloric acid and then extracted three times with diethyl ether. The combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography over silica using 5:1:0.05 hexane/ethyl acetate/formic acid. Yield: 163 mg (45%). For NMR data see Table 1.

RESULTS AND DISCUSSION

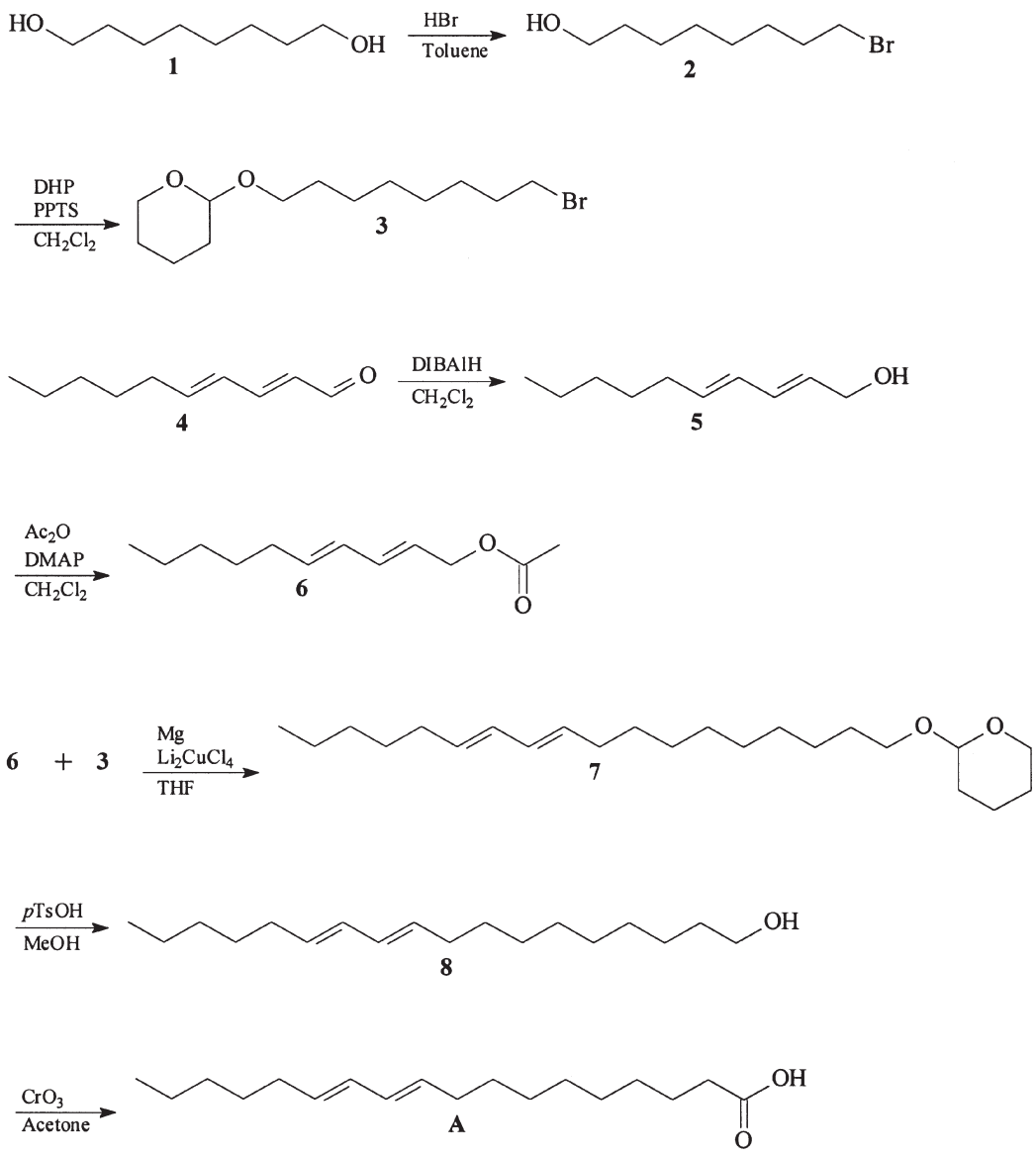
Because of the difficulties in the isolation of single isomers from more or less complex mixtures, stereoselective syntheses of octadecadienoic acids with conjugated double bonds, also called CLA, are advantageous as they do not entail further purification procedures. Several strategies are used in the preparation of stereochemically pure CLA. The approach of choice depends largely on the availability of the corresponding starting material and yields, as well as on demands concerning absolute amounts and (stereochemical) purity of the product. In this paper we describe four different syntheses, three of which we followed in detail to evaluate the reactions with respect to the above-mentioned criteria.

A simple method (Scheme 1A) involves chain elongation of an appropriate dienic compound with a Grignard reagent using Schlosser–Fouquet conditions (10). If the Grignard reagent is prepared from a protected ω-halogen alcohol, the resulting protected dienol needs to be converted to the corresponding dienoic acid. Yields of the reaction are good, and the stereochemical purity of the product is the same as that of the educt.

A second sequence (Scheme 1B) involves the construction of an enyne system followed by stereoselective hydrogenation of the triple bond. The enyne may be formed upon Sonogashira reaction of an (*E*)- or (*Z*)-configured vinyl halide with the anion of a terminal alkyne (11). Whereas the triple bond cannot be successfully transformed to an (*E*)-double bond, hydrogenation by using a copper-activated zinc catalyst (12) or a borane (13) generates a double bond with high (*Z*)-selectivity. Other methods, for example the reduction of alkynes by metallic sodium or lithium dissolved in liquid ammonia, also yield *trans*-alkenes, but the purity of the products would be insufficient for the synthesis of pure isomers. As a result, depending on the configuration of the vinyl halogenide and on the location of the additional terminal oxygen-containing functional group (either in the vinyl halide or in the alkyne), conjugated (*Z*,*Z*)-, (*Z*,*E*)-, and (*E*,*Z*)-systems may be produced. Yields of the reactions are good to excellent, but the stereochemical purity of the products is not constantly high. The coupling reaction is somewhat tricky with respect to stereoselectivity, and conditions must be carefully optimized and controlled. It should be mentioned that the (*Z*,*Z*)-configured dienes are always a bit unstable and prone to rearrangements.



SCHEME 1



SCHEME 2

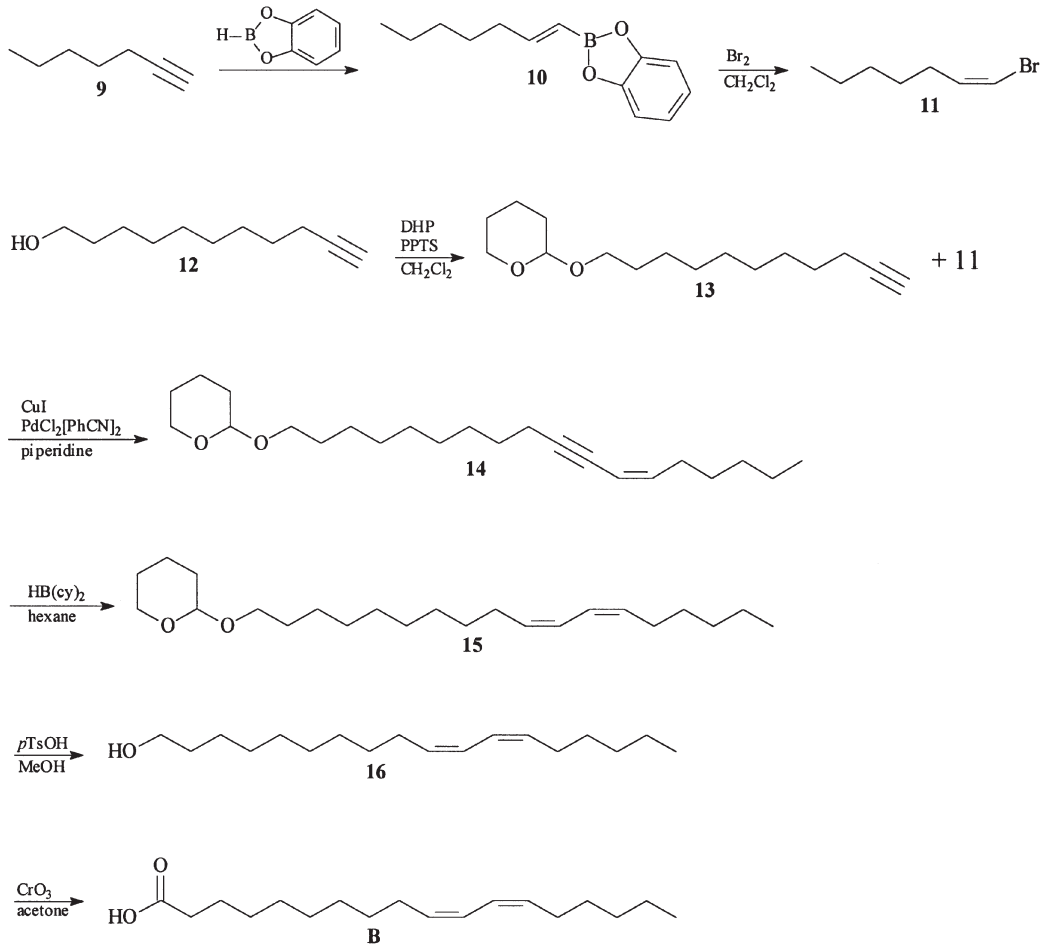
An alternative approach, also resulting in the formation of an enyne, would be a stereoselective Wittig reaction involving a propargylic aldehyde. A simpler reaction of a readily available (*E*)-2-alkenal with an appropriate Wittig reagent would directly yield a conjugated system (Scheme 1C). In our hands, yields of reactions were always good; however, the stereoselectivity was frequently found to be insufficient.

An elegant and versatile method for the generation of a conjugated double bond system is the Pd(0)-catalyzed coupling of a vinyl halide with a corresponding partner (Scheme 1D). The Suzuki reaction provides access to all four stereoisomers, as an (*E*)-or (*Z*)-1-alkenylboronic acid may be linked to an (*E*)- or (*Z*)-configured vinyl halide (14). While we could not constantly obtain very high yields, the stereoselectivity of the reaction was always excellent.

The first approach, a simple method for the synthesis of (10*E*,12*E*)-octadecadienoic acid **(A)** (Scheme 2), uses 1,8-octanediol (**1**) and commercially available (2*E*,4*E*)-decadienal (**4**). 1,8-Octanediol was converted to 8-bromo-1-octanol (**2**) using a liquid-liquid extractor to avoid bromination of both hydroxy groups. The free hydroxy group was then protected with 3,4-dihydropyran, yielding 8-(2′-tetrahydropyranyloxy)-1-bromooctane (**3**) as the first building block. The second building

TABLE 2
Yields and Purities of Synthesized Octadecadienols

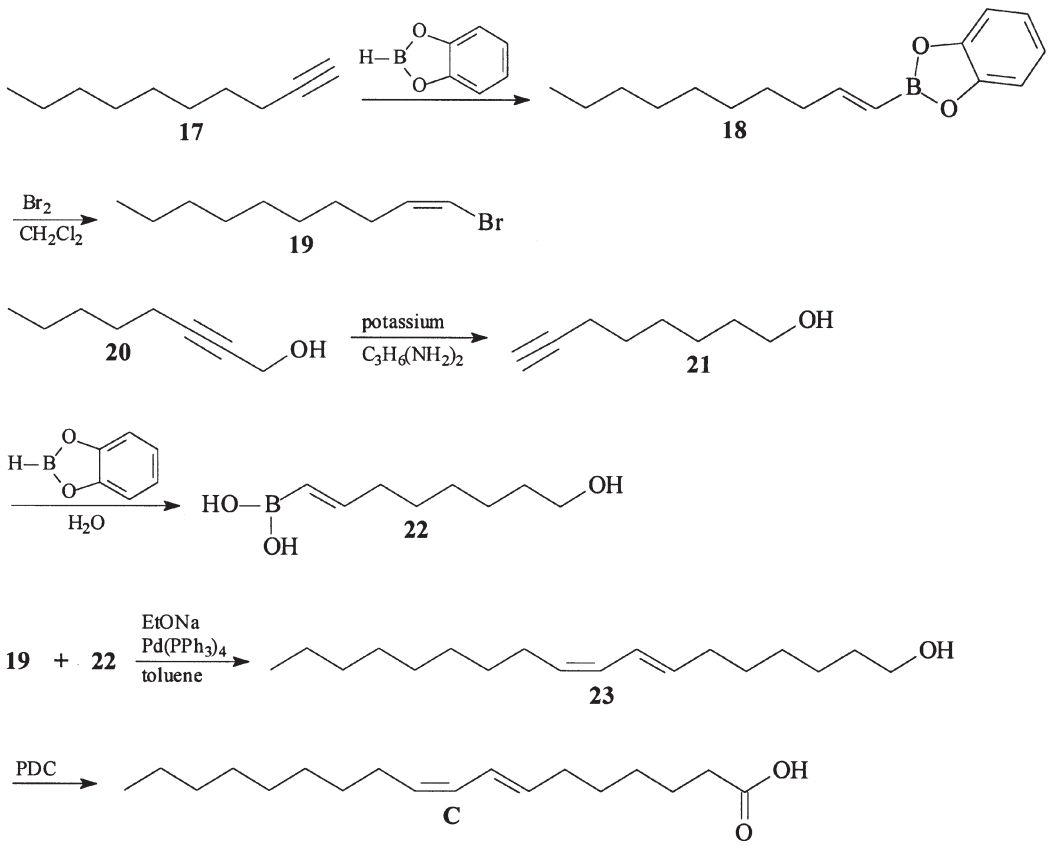
Compound	Overall yield (%)	Stereochemical purity (%)	Method
10*E*,12*E* **(A)**	40	93[a]	Diene elongation
10*Z*,12*Z* **(B)**	26	80	Enyne approach
7*E*,9*Z* **(C)**	12	98	Suzuki coupling



SCHEME 3

block was generated from (2*E*,4*E*)-decadienal, which was reduced to (2*E*,4*E*)-decadienol using diisobutylaluminum hydride. The hydroxy group was acetylated with acetic anhydride yielding (2*E*,4*E*)-decadienylacetate (**6**). For the coupling of these two building blocks, 8-(2′-tetrahydropyranyloxy)-1-bromoctane (**3**) was converted to the Grignard reagent, which was then added to a solution of (2*E*,4*E*)-decadienylacetate (**6**) and lithiumtetrachlorocuprate (10). The obtained protected alcohol (**7**) showing a conjugated (*E*,*E*)-configured double bond system was deprotected to yield (10*E*,12*E*)-octadecadienol (**8**), which was oxidized to (10*E*,12*E*)-octadecadienoic acid **A** with Jones reagent (15). Owing to dehydrobromination of the protected alkenyl bromide, 1-(2′-tetrahydropyranyloxy)-7-octene may be formed as a by-product during the coupling reaction with lithium tetrachlorocuprate. This impurity can be easily removed by liquid chromatography or by recrystallization of the acid from methanol. This method furnished (10*E*,12*E*)-octadecadienoic acid in a stereochemical purity of 93% (capillary GC, CP 8944, VF-5ms factor four WCOT; see Table 2).

The second approach, the synthesis of (10*Z*,12*Z*)-octadecadienoic acid **(B)**, proceeding *via* an enyne substructure (Scheme 3), requires (*Z*)-1-bromo-1-heptene (**11**) and 1-(2′-tetrahydropyranyloxy)-undec-10-yne (**13**) as the building blocks. (*Z*)-1-Bromo-1-heptene (**11**) was prepared by reaction of 1,3,2-benzodioxaborole with 1-heptyne (**9**), yielding the (*E*)-1-alkenylboronic acid ester **10**. The *trans*-configured double bond was reacted with bromine, which proceeded with *trans*-addition. The following elimination of the borone moiety and bromide produced the desired (*Z*)-1-bromo-1-heptene (16). Coupling this intermediate to protected 10-undecyn-1-ol yielded (*Z*)-1-(2′-tetrahydropyranyloxy)-octadec-10-yn-12-ene (**14**). The subsequent coupling was catalyzed by copper iodide and bis(benzonitrile)dichloropalladium(II), furnishing good yields with piperidine as the ideal solvent (11). The (*Z*,*Z*)-configured conjugated diene system was generated by hydrogenation of the triple bond using dicyclohexylborane (15). Stereoselective *syn*-addition of dicyclohexylborane followed by deprotection yielded (10*Z*,12*Z*)-octadecadienol (**16**). The latter was oxidized with Jones reagent to yield (10*Z*,12*Z*)-octadecadienoic acid **B**. This approach is suitable for isomers with at least one *Z*-configured double bond, because selective hydrogenation of the triple bond to the *E*-configured double bond is not possible. For the synthesis of (10*Z*,12*Z*)-octadecadienoic acid an overall yield of 20% with a stereochemical purity of 80% was achieved (capillary



SCHEME 4

GC, CP 8944, VF-5ms factor four WCOT, see Table 2). The major impurity was (10*Z*,12*E*)-octadecadienoic acid.

The third approach, a Suzuki coupling to (7*E*,9*Z*)-octadecadienoic acid (**C**) (Scheme 4), started with 1-decyne (**17**) and 7-octyn-1-ol (**21**), prepared from 2-octyne-1-ol (**20**) through Zipper reaction (17). To obtain the boronic acid (**22**), 7-octyne-1-ol was reacted with 1,3,2-benzodioxaborole using THF as the solvent. Hydrolysis to **22** was achieved by adding water and stirring for 2 h at room temperature (14). The second building block was prepared from 1-decyne (**17**), which was reacted with 1,3,2-benzodioxaborole to yield the (*E*)-1-alkenylboronic acid ester **18**. The *trans*-configured double bond was reacted with bromine, which proceeded with *trans*-addition. The following elimination of the borone moiety and bromide produced the desired (*Z*)-1-bromo-1-decene (**19**) (16). Coupling of the two building blocks was achieved with tetrakis triphenylphosphine palladium(0) using sodium ethanolate as base (14). The obtained (7*E*,9*Z*)-octadecadien-1-ol (**23**) was oxidized with pyridinium dichromate in DMF (18) yielding (7*E*,9*Z*)-octadecadienoic acid (**C**) in good yields. By following this method, all four geometrical isomers of

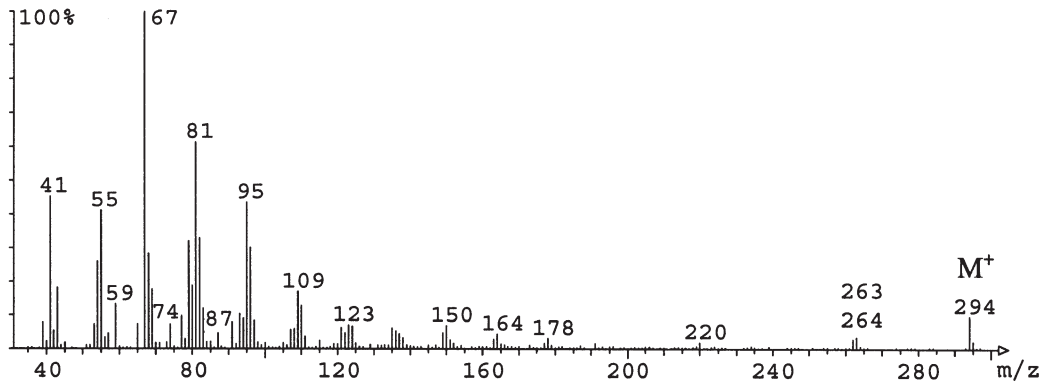


FIG. 1. Mass spectrum of methyl (10*E*,12*E*)-octadecadienoate.

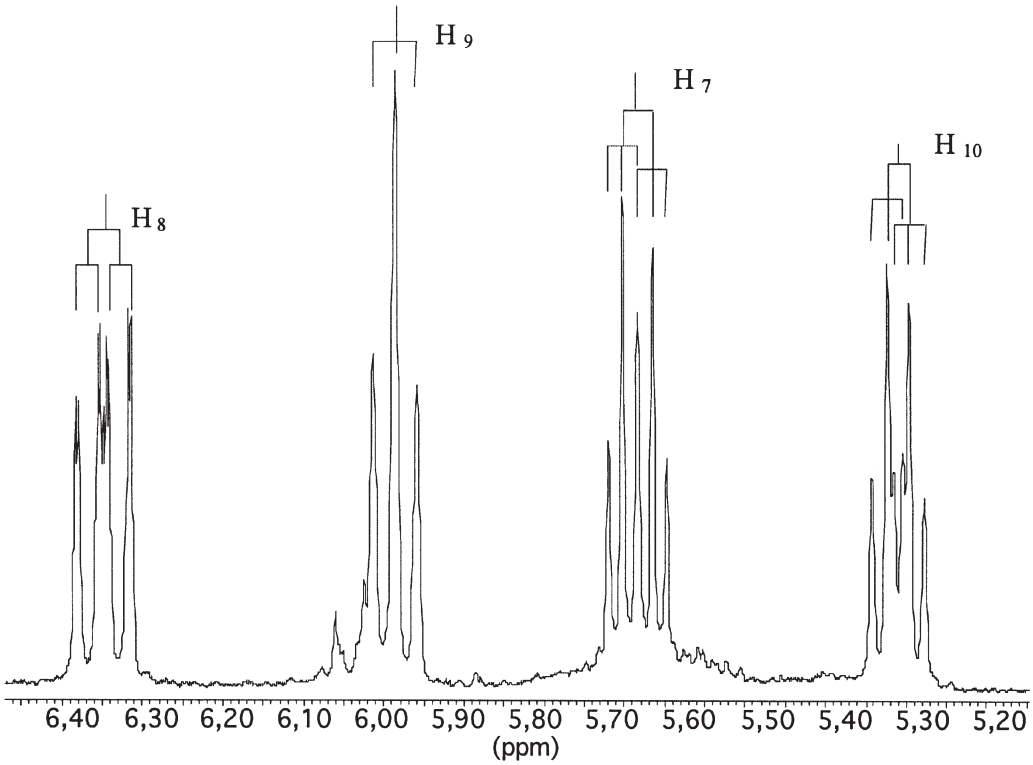


FIG. 2. ^{1}H NMR of olefinic protons of 7*E*,9*Z*-CLA.

octadecadienoic acid are accessible (14). For the synthesis of (7*E*,9*Z*)-octadecadienoic acid, an overall yield of 10% and a stereochemical purity of 98% was achieved (capillary GC, CP 8944, VF-5ms factor four WCOT, see Table 2).

In the context of our investigations, two different approaches for the oxidation of octadecadienols were followed: oxidation with Jones reagent (15) or with pyridinium dichromate (18). Oxidation with Jones reagent furnished low yields (<20%). Relatively large amounts of by-products were formed on decomposition of the chain. Several recrystallization steps were necessary for purification. Oxidation with pyridinium dichromate yielded the desired products in yields up to 45%, and lower amounts of by-products were formed. A third method proved to be even superior to PDC: During a two-step procedure, the alcohol is first oxidized to the aldehyde [e.g., Swern oxidation (19) or Dess–Martin oxidation (20)], which is then further oxidized with sodium chlorite (21). Although the synthesis of CLA involves an additional step, yields were higher and purification of the crude products was easier.

For the characterization of synthesized compounds, several methods were used. Mass spectra of methyl esters of dienoic acids may confirm the presence of a conjugated double bond system; however, they do not provide reliable information about the position or configuration of double bonds. As an example, the mass spectrum of methyl (10*E*,12*E*)-octadecadienoate is shown in Figure1. Mass spectra of methyl esters of stereoisomers look the same; the fragmentation pattern of positional isomers is also very similar.

TABLE 3
^{1}H NMR Signals of Olefinic Protons

Compound				
10*E*,12*E*	10-H	11-H	12-H	13-H
(A)	*m* 5.51–5.61	*m* 5.95–6.03	*m* 5.95–6.03	*m* 5.51–5.61
10*Z*,12*Z*	10-H	11-H	12-H	13-H
(B)	*m* 6.09–6.16	*m* 6.81–6.88	*m* 6.81–6.88	*m* 6.09–6.16
7*E*,9*Z*	7-H		8-H	9-H
10-H				
(C)	*dt* 5.64	*dd* 6.30	*dd* 5.93	*dt* 5.31
	J = 15.1/7.2 Hz	*J* = 15.1/11.0 Hz	*J* = 10.8/10.8 Hz	*J* = 10.9/7.6 Hz

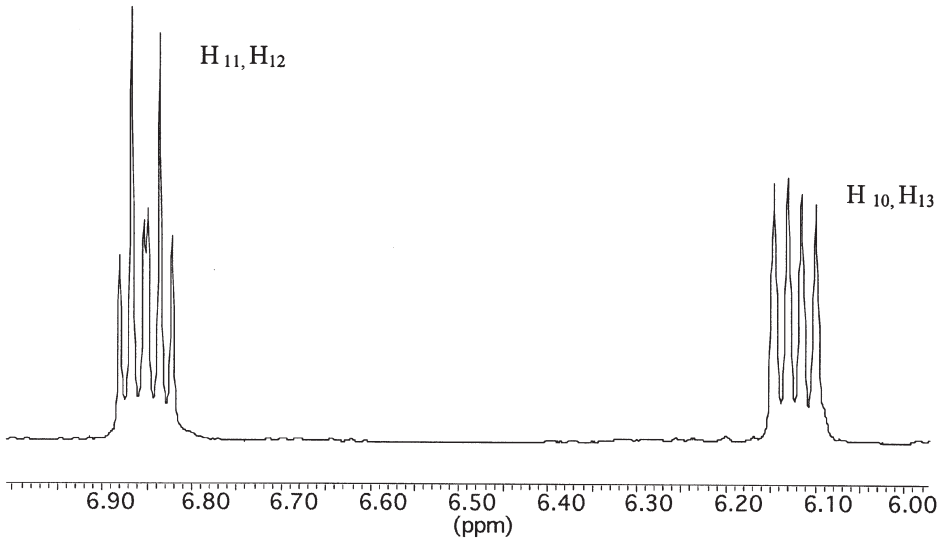


FIG. 3. ^{1}H NMR of olefinic protons of 10*Z*,12*Z*-CLA.

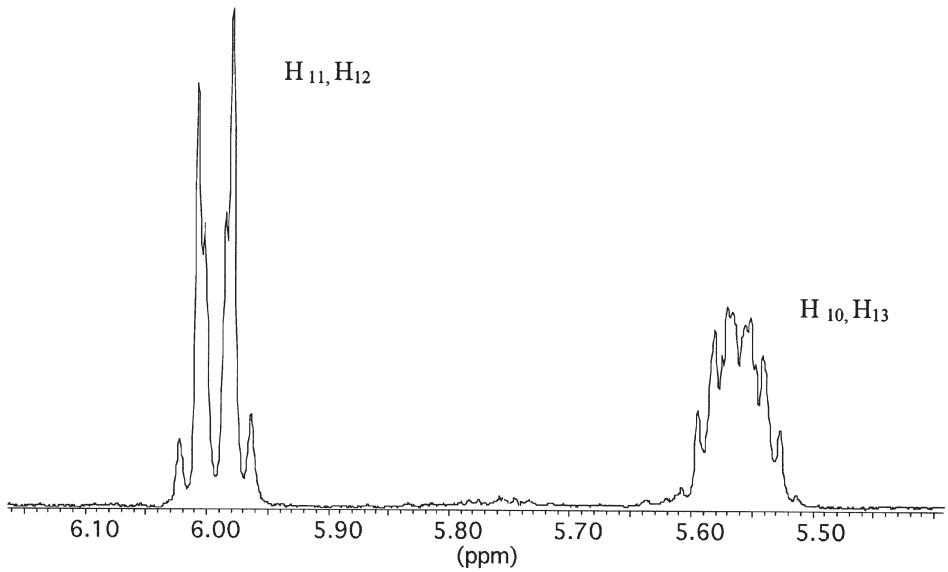


FIG. 4. ^{1}H NMR of olefinic protons of 10*E*,12*E*-CLA.

Information on the configuration of double bonds can be obtained from ^{1}H NMR spectra. Figure 2 shows the olefinic protons of 7*E*,9*Z*-CLA. Owing to the appearance of distinct signals for every olefinic proton, the conformation can be determined by investigation of the coupling constants (see Table 3). as *EE*- or *ZZ*-configured dienes represent symmetrical systems, attribution of configuration *via* coupling constants is very difficult or even impossible (Figs. 3, 4). In contrast, IR spectra provide valuable information to distinguish between these two stereoisomers (Fig. 5). For *EE*-configured isomers, a sharp absorption just below 1000 cm^{-1} can be seen. In contrast, *ZE*- or *EZ*-configured isomers show two smaller absorptions in the same region, whereas *ZZ*-configured isomers do not show a pronounced absorption between 900 and 1000 cm^{-1} (22).

ACKNOWLEDGMENTS

This work was carried out as part of the joint research project "Lipids and Phytosteroles in Food." Financial support was provided by the Deutsche Forschungsgemeinschaft.

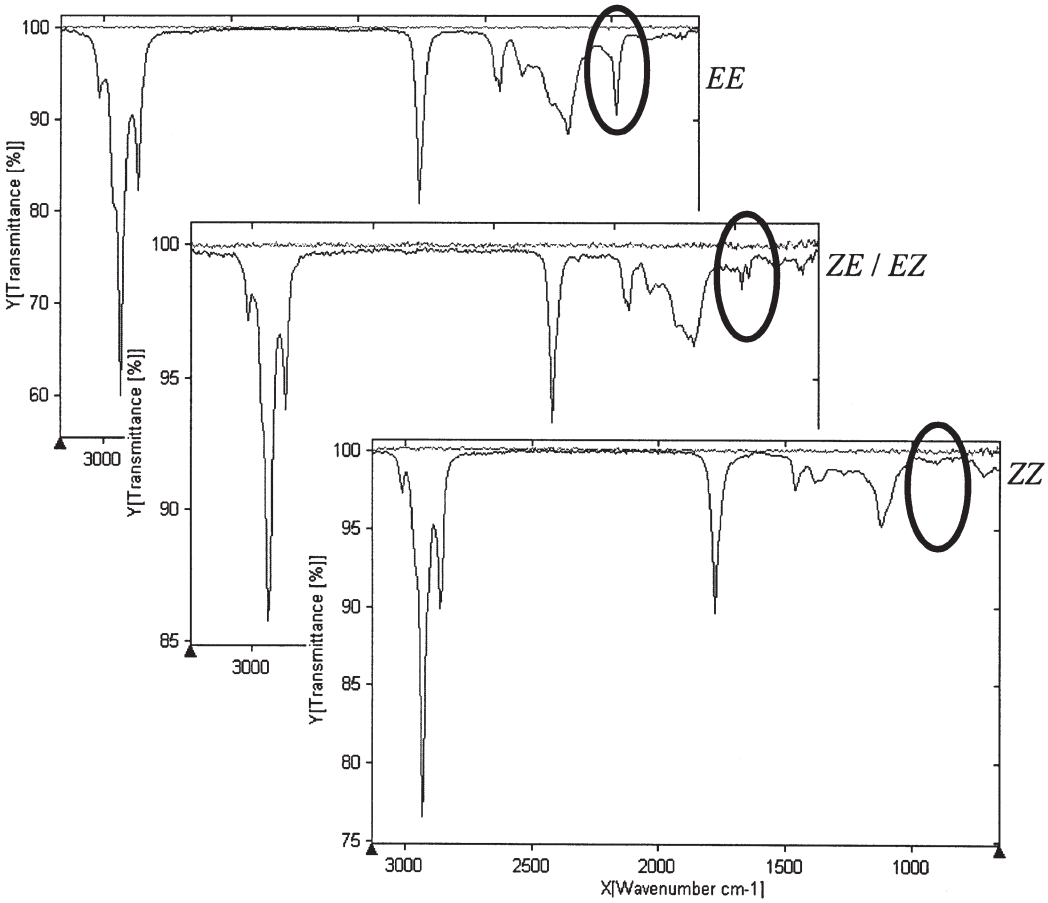


FIG. 5. IR spectra of CLA.

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Alterations of High Density Lipoprotein Subclasses in Obese Subjects

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ABSTRACT: The object of this study was to investigate the characteristics of lipid metabolism in obese subjects, with particular emphasis on the alteration of HDL subclass contents and distributions. A population of 581 Chinese individuals was divided into four groups (25 underweight subjects, 288 of desirable weight, 187 overweight, and 45 obese) according to body mass index (BMI). Apoprotein A-I (apoA-I) contents of plasma HDL subclasses were determined by 2-D gel electrophoresis associated with an immunodetection method. The concentrations of TG and the apoA-I content of pre-β_1-HDL were significantly higher ($P < 0.01$ and $P < 0.01$, respectively), but the levels of HDL cholesterol, and the apoA-I contents of HDL_{2a} and HDL_{2b} were significantly lower ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively) in obese subjects than in subjects having a desirable weight. Moreover, with the elevation of BMI, small-sized pre-β_1-HDL increased gradually and significantly, whereas large-sized HDL_{2b} decreased gradually and significantly. Meanwhile, the variations in HDL subclass distribution were more obvious with the elevation of TG levels in obese as well as overweight subjects. In addition, Pearson correlation analysis revealed that BMI and TG levels were positively correlated with pre-β_1-HDL but negatively correlated with HDL_{2b}. Multiple regression analysis also showed that TG concentrations were associated independently and positively with high pre-β_1-HDL and independently and negatively with low HDL_{2b} in obese and overweight subjects. The HDL particle size was smaller in obese and overweight subjects. The shift to smaller size was more obvious with the elevation of BMI and TG, especially TG levels. These observations, in turn, indicated that HDL maturation might be abnormal, and reverse cholesterol transport might be impaired.

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Epidemiologic data have firmly established that HDL evaluated by its cholesterol (C) content is inversely correlated with the incidence and prevalence of atherosclerosis (1,2). Low HDL-C level has been a significant independent risk factor for atherosclerosis in many prospective human population studies. (3,4). This antiatherogenic action of HDL is probably related to reverse cholesterol transport (RCT), by which excess peripheral cholesterol is returned to the liver (5). In recent years, changes in HDL subclass distribution have been considered as more closely correlated with atherosclerosis than low plasma HDL-C levels (6). Many investigators believe that measuring HDL subclasses provides additional information about atherosclerotic risk as well as about the underlying physiological mechanism(s) responsible for that risk. Atger *et al.* (7) considered that the alteration of plasma HDL subclasses might be an important marker for susceptibility to the development of coronary heart disease (CHD).

Differences in the quantitative and qualitative content of lipids, apolipoproteins (apo), enzymes, and lipid transfer proteins result in the various HDL subclasses, which are characterized by shape, density, size, charge, and antigenicity (8). Subclasses of HDL can be separated by zonal (9) or single-spin vertical ultracentrifugation (10), heparin-magnesium precipitation (11), NMR spectroscopy (12), or 1- and 2-D PAGE. Using 2-D gel electrophoresis (13,14) and subsequent immunoblotting, HDL can be subdivided into large- (HDL_{2a} and HDL_{2b}) and small-sized subclasses (pre-β_1-HDL, HDL_{3c}, HDL_{3b}, and HDL_{3a}) and pre-β_2-HDL (15). The smallest pre-β_1-HDL picks up free cholesterol efficiently from endothelial cell membranes, then is transformed by the activity of LCAT, which catalyzes cholesterol ester formation from free cholesterol and lecithin-derived FA on the surface of HDL particles. With the further participation of LCAT and other specific plasma factors, such as hepatic lipase (HL), CETP, and the phospholipids transfer protein (PLTP), cholesterol ester is concentrated into the center of the lipoprotein molecule; HDL particles are transformed from nascent lipid-poor pre-β_1-HDL to mature spherical HDL_2. It has been postulated that RCT indeed is the metabolic process by which nascent pre-β_1-HDL converts to mature HDL_2, following the route of pre-β_1-HDL $\rightarrow$ pre-β_2-HDL $\rightarrow$ pre-β_3-HDL $\rightarrow$ HDL_3 $\rightarrow$ HDL_2. Owing to the important role of RCT in maintaining cholesterol homeostasis and in antiatherosclerosis, the HDL maturation process and distributions of HDL subclasses may directly influence the atherogenic progress (7,16,17).

Obesity is a major risk factor for CHD (18) and is also associated with an atherogenic lipoprotein profile. Low levels of

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Abbreviations: ApoA-I, apoprotein A-I; BMI, body mass index; CE, cholesteryl ester; CHD, coronary heart disease; CM, chylomicrons; HDL-C, HDL cholesterol; HL, hepatic lipase; LDL-C, LDL cholesterol; LPL, lipoprotein lipase; PLTP, phospholipids transfer protein; RCT, reverse cholesterol transport; SRC, standardized regression coefficient; TC, total cholesterol.

plasma HDL-C and high levels of plasma TG are commonly found in obese subjects (19). The relationship of a low HDL-C concentration to an increased risk of CHD has become a widely accepted concept. Data from the Québec Cardiovascular Disease Study (20) have shown that low HDL-C and high TG concentrations were associated with a substantial increase in the risk of CHD in obese subjects. Likewise, epidemiological studies provide increasing evidence that plasma TG level is an important independent risk indicator of CHD (21,22). On this basis, the aim of the present study was to investigate lipid metabolism in obese subjects, especially examining differences in HDL subclass distributions and content, which may help us understand the pathogenesis of atherosclerosis in obese subjects.

MATERIALS AND METHODS

Subjects. Five hundred eighty-one Chinese subjects aged 33–78 yr (431 were from West China University of Medical Science, Sichuan University, and Sichuan Normal University, in Chengdu, Sichuan Province, PR China; 150 were from Nan Hua University in Hengyang, Hunan Province, PR China) were recruited to participate in a study examining plasma lipid and apo concentrations. The study protocol was approved by an ethics committee, and all subjects gave informed consent. Exclusion criteria included the presence of (i) nephritis, diabetes mellitus, hypothyroidism, hepatic impairment; (ii) major cardiovascular event (myocardial infarction, severe or unstable angina pectoris, surgery), stroke; (iii) consumption of lipid-altering medications in the previous month; (iv) presence of chylomicrons (CM) in the refrigerator test; (v) history of smoking, drug, or alcohol abuse.

Body mass index (BMI) was calculated as the ratio of weight (kg) to the square of height (m^2). Finally, according to the recommended criterion (23) for BMI of the Working Group on Obesity in China (WGOC) under the support of International Life Sciences focal point in China (23), the subjects were divided into four groups: underweight, of desirable weight, overweight, and obese.

Twenty-five subjects with BMI < 18.5 kg/m^2 were assigned to the underweight group, 288 with BMI 18.5–24 kg/m^2 were assigned to the group having a desirable weight, 187 with 24 < BMI ≤ 28 kg/m^2 were considered overweight, and 45 with BMI > 28 kg/m^2 were defined as obese.

To investigate the impact of TG levels on the distributions of HDL subclasses, we divided obese and overweight subjects into four groups according to plasma TG levels, that is, normal (<1.69 mmol/L), borderline-high (1.69–2.25 m mol/L), high (2.26–5.64 m mol/L), and very high (≥5.65 m mol/L), and observed alterations of the HDL subclasses.

The TG levels selected for classification generally conform to the Adult Treatment Panel III guidelines (24).

Specimens. Blood specimens were drawn after a 12-h overnight fast into EDTA-containing tubes. Plasma was separated at room temperature within 1–2 h. Plasma was stored at 4°C and used within 24 h for lipid and apo analyses. An aliquot of plasma was stored at –70°C for the determination of HDL subclasses.

Plasma lipid and apo analyses. Plasma TG, total cholesterol (TC), and HDL-C were measured by standard techniques. TG and TC were determined with enzymatic kits (Beijing Zhongshen Biotechnological Corporation, Beijing, P.R. China). HDL-C was determined after precipitation of apoB-containing lipoproteins by phosphotungstate/magnesium chloride. When TG was less than 4.52 m mol/L, LDL-cholesterol (LDL-C) was calculated using the Friedwald formula. When TG exceeded 4.52 m mol/L, LDL-C was determined with enzymatic kits (Beijing Zhongshen). Plasma apoA-I, B100, C-II, C-III, and E were determined by radial immunodiffusion methods using kits developed at the Apolipoprotein Research Laboratory, West China Medical Center, Sichuan University.

HDL subclass analyses. HDL subclass distribution was determined with 2-D gel electrophoresis associated with an immunodetection method as described previously (14). In brief, 10 μL of plasma was applied to 0.7% agarose gel in the first dimension. After electrophoretic separation of lipoproteins in agarose gel, they were further separated by electrophoresis in 2–30% nondenaturing polyacrylamide gradient gel in the second dimension. To determine HDL subclasses, western blotting was performed after electrophoresis, using horseradish peroxidase-labeled goat anti-human apoA-I–IgG. The relative concentration of each HDL subclass was calculated as the percentage of plasma apoA-I according to the density of each spot. HDL particle sizes were calibrated using a standard curve that included BSA, ferritin, and thyroglobulin (Pharmacia, Hong Kong, P.R. China). Then the relative percent concentration of each HDL subclass was multiplied by the apoA-I concentrations in each individual, respectively, to provide the relative concentration of each HDL subclass of apoA-I (mg/L, apoA-I in the subclasses). The interassay CV of relative concentration of pre-β_1-HDL, pre-β_2-HDL, HDL_{3c}, HDL_{3b}, HDL_{3a}, HDL_{2a}, and HDL_{2b} in plasma samples were 9.4, 9.8, 4.9, 6.2, 7.3 11.1, and 7.9%, respectively ($n = 5$).

Statistical analysis. Numerical data are expressed as mean ± SD. Plasma TG was logarithmically transformed owing to its skewed distribution. Grouped data were compared by ANOVA. Differences were considered statistically significant at $P < 0.05$. Linear correlation (Pearson) and multiple regression analysis were used to test correlations between BMI, lipid, and HDL subclasses. All statistical analysis was performed using the statistical package SPSS 13.0 (SPSS Inc., Chicago, IL).

RESULTS

Concentrations of plasma lipids and apo. As shown in Table 1, when comparing subjects having a desirable weight with those who were obese, values for BMI; concentrations of TG, $apoB_{100}$, apoC-II, apoC-III; and the ratios of TG/HDL-C and TC/HDL-C were significantly higher whereas the levels of HDL-C and apoA-I were significantly lower. Underweight subjects showed the opposite trend in the parameters just mentioned.

ApoA-I contents of HDL subclasses. Figure 1 shows the distributions of HDL subclasses for representative subjects from the group having desirable weights (normolipidemia) (A) and

TABLE 1
Concentrations of Plasma Lipids and Apolipoproteins[a]

	Underweight (BMI: ≤18.5 kg/m^2, n = 25)	Desirable (BMI: 18.5–24 kg/m^2, n = 288)	Overweight (BMI: 24–28 kg/m^2, n = 187)	Obese (BMI: >28 kg/m^2, n = 45)
Age (yr)	57.5 ± 9.6	56.1 ± 9.8	57.5 ± 9.0	55.5 ± 7.6
BMI (kg/m^2)	17.5 ± 0.6[e]	21.9 ± 1.4	25.4 ± 1.1[e]	29.7 ± 1.9[e]
TG (mmol/L)[a]	1.4 ± 0.4[c]	2.1 ± 0.6	2.6 ± 0.8[d]	3.4 ± 1.1[d]
TC (mmol/L)	5.8 ± 0.6	5.6 ± 0.7	5.4 ± 0.8	5.7 ± 0.8
LDL-C (mmol/L)	3.5 ± 0.9	3.3 ± 1.0	3.1 ± 0.9	3.2 ± 0.8
HDL-C (mmol/L)	1.7 ± 0.7[d]	1.3 ± 0.4	1.1 ± 0.3[d]	1.0 ± 0.3[d]
TG/HDL-C	0.9 ± 0.3[c]	1.6 ± 0.4	2.4 ± 0.6[d]	3.4 ± 0.8[d]
TC/HDL-C	3.4 ± 0.8[d]	4.3 ± 1.0	4.9 ± 1.5	5.8 ± 1.4[d]
apoA-I (mg/L)	1363.0 ± 202.1	1275.7 ± 211.9	1202.8 ± 185.8[c]	1196.5 ± 178.5[c]
apoB100 (mg/L)	842.3 ± 140.4	920.4 ± 240.5	900.9 ± 206.1	993.1 ± 167.0[d]
apoC-II (mg/L)	52.0 ± 14.7[d]	69.7 ± 22.1	69.9 ± 23.6	80.7 ± 26.1[d]
apoC-III (mg/L)	119.1 ± 28.5[d]	157.4 ± 60.2	165.7 ± 61.4	188.5 ± 72.4[d]
apoE (mg/L)	46.0 ± 11.2	53.6 ± 19.8	55.7 ± 22.5	59.9 ± 17.9

[a]Values are expressed as mean ± SD. Abbreviations: BMI, body mass index; TG, triglyceride; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG/HDL-C, the ratio of TG to HDL-C; TC/HDL-C, the ratio of TC to HDL-C; apo, apolipoprotein.
[b]Student t-test on $\log_e$-transformed data.
[d]$P < 0.05$
[e]$P < 0.01$
[f]$P < 0.001$ vs. group having desirable weights.

from the obese (B) group. In the obese subjects, spots for the small-sized subclasses (pre-β_1-HDL, HDL_{3a}, HDL_{3b}, and HDL_{3c}) were larger, whereas large-sized HDL_2 (HDL_{2a} and HDL_{2b}) had spots that were smaller in comparison with the normolipidemic subjects. Moreover, quantification of the HDL subclasses confirmed this observation. Data in Table 2 indicate that the concentrations of small-sized pre-β_1-HDL were significantly higher, whereas the concentrations of large-sized HDL_{2b} were significantly lower in both overweight and obese subjects, and HDL_{2a} were significantly lower only in obese

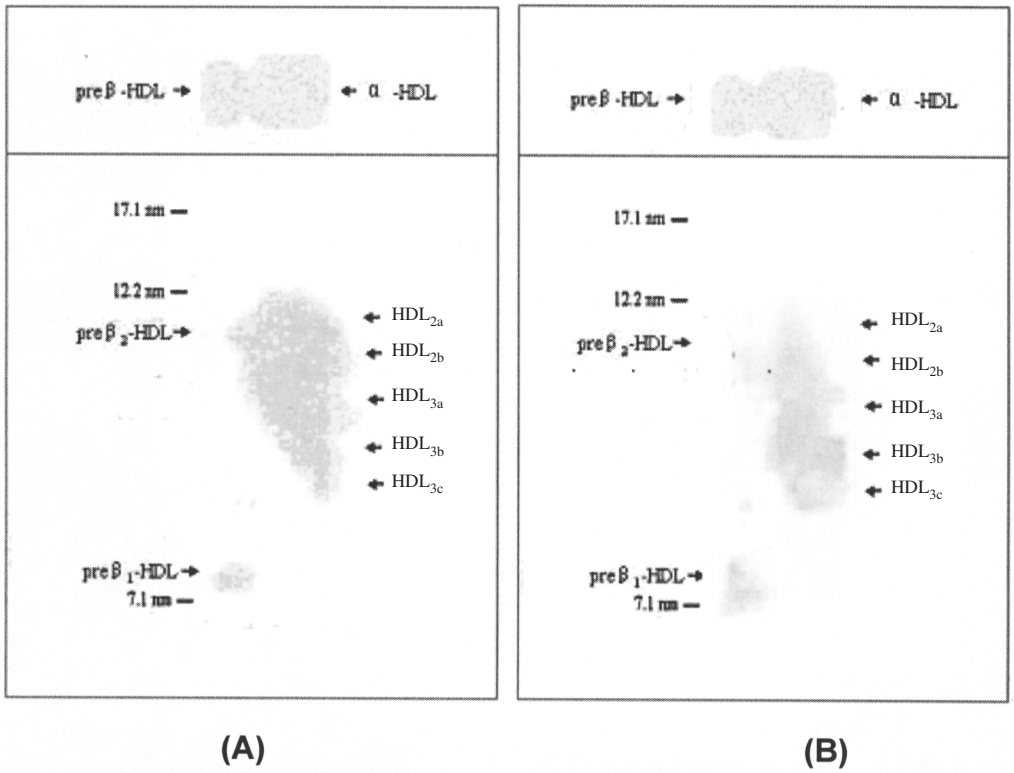

FIG. 1. Electrophoretic comparisons of apoprotein A-I (apoA-I)-containing HDL subclasses. HDL subclasses were separated by nondenaturing 2-D gel electrophoresis and immunodetection with a goat antihuman apoA-I–IgG labeled with horseradish peroxidase. (A) Samples from subjects having desirable weights (normolipidemic). (B) Obese subjects.

TABLE 2
The Relative Contents of apoA-I in the various HDL Subclasses[a]

	Underweight (BMI: ≤18.5 kg/m^2, n = 25)	Desirable (BMI: 18.5–24 kg/m^2, n = 288)	Overweight (BMI: 24–28 kg/m^2, n = 187)	Obese (BMI: >28 kg/m^2, n = 45)
Pre-β_1-HDL	101.2 ± 33.0	110.7 ± 35.1	124.6 ± 40.2[b]	135.9 ± 43.1[c]
Pre-β_2-HDL	65.0 ± 20.1	60.1 ± 19.3	56.2 ± 18.3	59.1 ± 18.9
HDL_{3c}	74.0 ± 23.1	77.3 ± 25.2	70.4 ± 22.6	71.9 ± 21.7
HDL_{3b}	177.3 ± 45.6	152.8 ± 39.7	140.2 ± 32.8	147.9 ± 43.1
HDL_{3a}	307.1 ± 78.2	295.9 ± 73.7	245.3 ± 67.6	242.1 ± 65.5
HDL_{2a}	292.1 ± 77.3[c]	258.2 ± 73.8	260.1 ± 58.1	233.0 ± 51.8[b]
HDL_{2b}	346.3 ± 96.6[b]	320.4 ± 95.2	304.0 ± 81.7[b]	248.6 ± 56.9[c]

[a]Values are expressed as mean ± SD. Abbreviations: Pre-β_1-HDL, pre-β_2-HDL, HDL_{3c}, HDL_{3b}, HDL_{3a},HDL_{2a}, HDL_{2b}: HDL subfractions; for other abbreviations see Table 1.
[b]$P < 0.05$.
[c]$P < 0.01$, vs. group having desirable weights.

subjects vs. subjects having a desirable weight. However, compared with those having desirable weights, the concentrations of large-sized HDL_{2a} and HDL_{2b} were significantly higher in underweight subjects.

Frequency distributions of pre-β_1-HDL and HDL_{2b} in subjects having desirable weights and in obese subjects. The general profile of frequency distributions of pre-β_1-HDL (Fig. 2) and HDL_{2b} (Fig. 3) levels of obese subjects shifted to the right and left, respectively, compared with those of subjects having desirable weights, which indicated that plasma HDL particles tend to be smaller in obese subjects.

ApoA-I contents of HDL subclasses at different plasma TG levels in obese and overweight subjects. Table 3 showed that, with the elevation of TG levels, apoA-I contents of small-sized particles (especially pre-β_1-HDL and HDL_{3a}) gradually and significantly increased, whereas large-sized HDL_{2a} and HDL_{2b} gradually and significantly decreased.

Correlation coefficients between apoA-I contents of HDL subclasses, plasma lipids, and BMI in overweight and obese subjects. Correlation analysis revealed (Table 4) that BMI and plasma levels of TG were significantly and positively correlated with small-sized pre-β_1-HDL and negatively correlated with large-sized HDL_{2b}; conversely, levels of HDL-C showed a negative correlation with pre-β_1-HDL and a positive correlation with HDL_{2b} in obese and overweight subjects. Of note, TC showed a positive correlation only with pre-β_1-HDL.

Multiple regression analysis between apoA-I contents of HDL subclasses, plasma lipids, and BMI in overweight and obese subjects. To obtain a better understanding of the determinants of HDL subclass distribution in obese subjects, we performed stepwise multivariate regression analyses using apoA-I contents of pre-β_1-HDL and apoA-I contents of HDL_{2b} as dependent variables (Table 5). The pre-β_1-HDL was significantly and independently predicted by TG [standardized regression coefficient (SRC) 0.620, $P < 0.001$. The adjusted r^2 for this model, 0.304, explained about 30% of the variation in pre-β_1-HDL. In

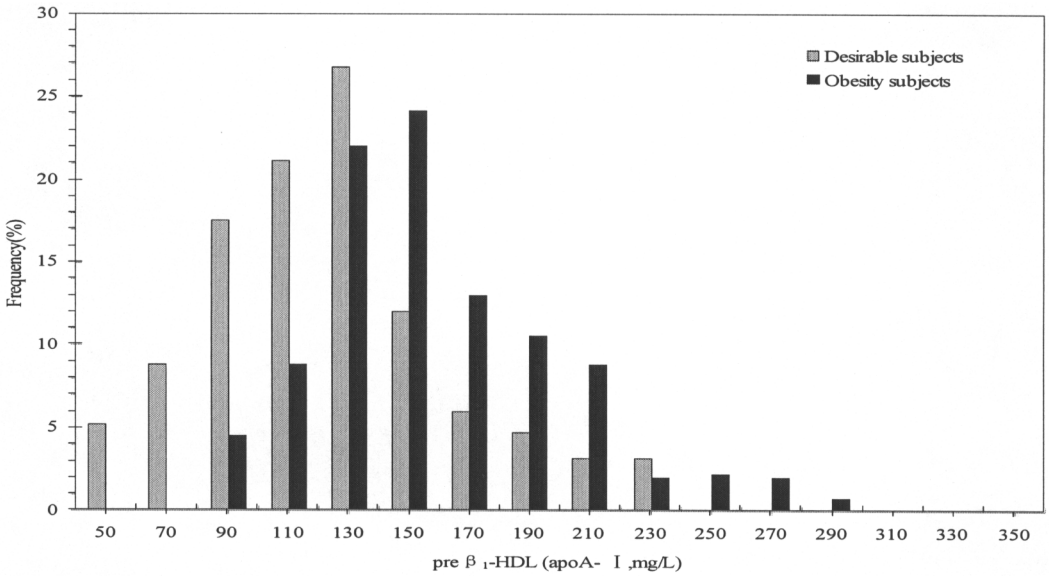

FIG. 2. Frequency distribution of pre-β_1-HDL levels in subjects having desirable weights and in obese subjects.

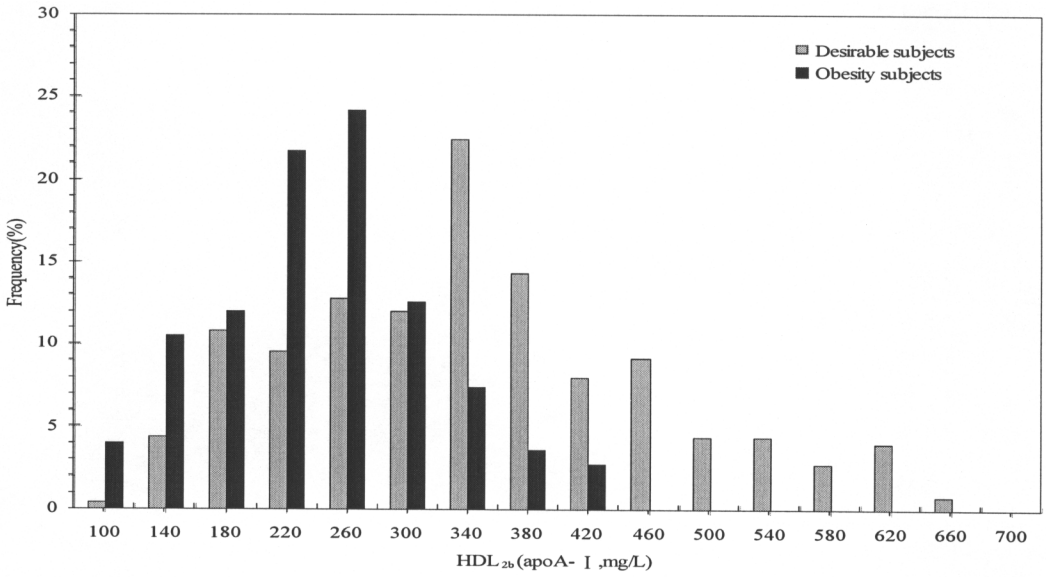

FIG. 3. Frequency distribution of HDL_{2b} levels in subjects having desirable weights and in obese subjects.

similar analyses with HDL_{2b} as the dependent variable, TG (SRC = –0.515, $P < 0.001$). The adjusted r^2 for this model was 0.307; however, BMI was not related to pre-β_1-HDL or HDL_{2b} and was removed from the final model.

DISCUSSION

The obese state accentuates the known risk factors for atherosclerotic disease. The prevalence of individuals who are overweight or obese is increasing rapidly in the Chinese population. The causes of obesity are complex. The consensus is that people become obese as result of a lifestyle consisting of low levels of physical activity and that consumption of excess calories plays a dominant role in the development of obesity. It is well known that obesity, especially central obesity (when the main deposits of fat are localized around the abdomen and upper body), is associated with dyslipidemia characterized by increased TG and decreased HDL-C (25–28). Several studies demonstrated the influence of obesity on lipoproteins and apo, but few investigations to date focused on relationship between obesity and HDL subclass distribution. Our laboratory had investigated the apoA-I contents of HDL subclass distribution in a Chinese population that was hyperlipidemic and experiencing endogenous hypertriglyceridemia by 2-D gel electrophoresis in association with an immunodetection method (15,29–32). Although there were some minor differences of HDL subclass distribution in hyperlipidemic subjects having different phenotypes, the common tendency was of increased small-sized HDL and decreased large-sized HDL particles. In the present study, we mainly investigated the characteristic of lipid metabolism, especially the distribution profile of HDL subclasses in obese subjects.

We observed that the concentration of TG and the contents of apoA-I of small-sized pre-β_1-HDL were significantly higher, but the concentration of HDL-C and the contents of apoA-I of large-sized HDL_{2a} and HDL_{2b} were significantly lower in obese subjects compared with subjects having a desirable weight. The results indicate that maturation metabolism of HDL might be ab-

TABLE 3
The apoA-I Contents[a] of HDL Subclasses at Different TG Levels in Subjects with BMI > 24 kg/m^2

	Normal TG (<1.69 mmol/L) (n = 65)	Borderline-high TG (1.69–2.25 mmol/L) (n = 33)	High TG (2.26–5.64 mmol/L) (n = 119)	Very high TG (≥5.65 mmol/L) (n = 15)
Pre-β_1-HDL	71.3 ± 21.1	88.6 ± 37.4[b]	126.1 ± 44.3[d]	171.5 ± 46.9[d]
Pre-β_2-HDL	54.0 ± 19.1	48.1 ± 16.8	59.9 ± 20.1	62.8 ± 18.4
HDL_{3c}	70.2 ± 30.6	70.6 ± 30.4	70.9 ± 27.9	71.7 ± 28.2
HDL_{3b}	129.1 ± 42.5	142.9 ± 46.2	145.6 ± 50.3	179.5 ± 36.2[d]
HDL_{3a}	238.5 ± 43.8	287.8 ± 76.2[c]	289.9 ± 82.0[c]	332.4 ± 80.1[d]
HDL_{2a}	290.9 ± 70.4	241.2 ± 85.1[c]	226.4 ± 66.2[d]	191.7 ± 45.6[d]
HDL_{2b}	365.8 ± 87.8	335.3 ± 76.7[c]	259.4 ± 97.3[c]	178.3 ± 50.3[d]

[a]Values are expressed as mean ± SD (mg/L). For abbreviations see Tables 1 and 2.
[b]$P < 0.05$.
[c]$P < 0.01$.

TABLE 4
Correlation Coefficients Between Plasma Lipids, BMI and apoA-I Contents of HDL Subclasses[a] in Subjects with BMI > 24 kg/m^2, (n = 232)

	Pre-β_1-HDL	Pre-β_2-HDL	HDL_{3c}	HDL_{3b}	HDL_{3a}	HDL_{2a}	HDL_{2b}
BMI	0.383[b]	0.037	0.271[b]	0.210	0.026	–0.171	–0.303[b]
TG	0.521[b,*]	0.127	0.120	0.185	0.189	–0.361[b]	–0.513[b]
TC	0.282[b]	0.060	0.102	0.124	0.062	–0.109	–0.119
LDL-C	0.212	0.095	0.027	0.061	–0.083	–0.108	–0.254
HDL-C	–0.319[b]	–0.024	–0.287[b]	–0.014	–0.031	0.401[b]	0.434[b]

[a]For abbreviations see Tables 1 and 2.
[b]$P < 0.01$.

TABLE 5
Multiple Regression Analysis Between apoA-I Contents of Pre-β_1-HDL and HDL_{2b} and Plasma Lipids for Subjects Having BMI > 24 kg/m^2, (n = 232)

		Unstandardized coefficients		Standardized coefficients		
		β	Standard error	β	t	p
Preβ_1-HDL	TG	19.201	1.991	0.620	9.646	0.000
	LDL-C	12.441	3.388	0.236	3.673	0.000
HDL_{2b}	TG	–31.146	3.420	–0.515	–9.106	0.000
	HDL-C	96.285	21.010	0.313	4.583	0.000
	TC	–15.369	7.119	–0.134	–2.159	0.01

normal in obese subjects. The mechanism for the alteration of HDL subclass distribution in the obese subjects may be related to the several lipoprotein-modifying plasma enzymes: lipoprotein lipase (LPL), HL, LCAT, CETP, and others. Many researchers have demonstrated that impaired LCAT and LPL activities are associated with high plasma TG levels (33,34). LPL plays an important role in hydrolyzing TG transported in CM and VLDL particles (34). When catabolized by LPL, CM and VLDL release TG, TC, phospholipids, apoA-I, and apo C. Subsequent binding of these products to HDL_3 results in formation of HDL_2 particles. LCAT may convert unesterified cholesterol to cholesterol ester and promote the conversion of pre-β_1-HDL and HDL_3 to HDL_2 (33). Thus, reduced plasma LPL and LCAT activity leads to the reduction of HDL_2. Likewise, increased HL and CETP activity are associated with higher plasma TG levels (35–37). HL promotes the conversion of HDL_2 to HDL_3 *via* hydrolysis of its TG and phospholipids (35). CETP mediates exchange of TG and cholesteryl ester (CE) between VLDL-TG, LDL-TG, and HDL-CE, resulting in TG enrichment of HDL (36,37). The subsequent hydrolysis of transferred TG in HDL_2 levels leads to the formation of small HDL particles. Consequently, enhanced HL and CETP activities promote the conversion of HDL_2 to HDL_3, hydrolyze excess surface phospholipids, and lead to the dissociation of apoA-I from HDL_2; the latter may generate much of the small-sized pre-β_1-HDL. Most studies have demonstrated increased CETP activity increased and decreased PLTP activity in subjects with low HDL-C levels. PLTP can transfer phospholipids (surface remnants) from TG-enriched lipoproteins onto HDL (38); hence, it favors the formation of larger-sized HDL particles. Therefore, reduction of HDL-C and elevation of TG resulted in the marked increase in small-sized HDL subclasses and decrease in large-sized HDL subclasses.

To investigate the influence of plasma TG levels on the distributions of HDL subclasses, subjects with BMI > 24 kg/m^2 were separated into four subgroups according to the plasma TG levels identified in this study. We observed that with increased plasma TG levels, apoA-I contents of small-sized HDL particles (especially pre-β_1-HDL and HDL_{3a}) increased too, but those of large-sized HDL particles decreased, which suggested that the particle size of HDL tended to shift to smaller size, with the trend being more obvious with the increase of plasma TG levels in obese and overweight subjects. Meanwhile, compared with subjects with normal TG levels, an increase (24, 77, 140%) in pre-β_1-HDL and a decrease (8.9, 29, 52%) in HDL_{2b} were found in those with borderline high, high, and very high TG levels in the obese and overweight groups. These findings suggest that plasma TG levels have a significant effect on the distribution of HDL subclasses, which is in accordance with our previous finds on endogenous hypertriglyceridemic subjects (29). The Pearson correlation analysis confirmed that TG concentration was positively correlated with small-size pre-β_1-HDL and negatively correlated with large-size HDL_{2a} and HDL_{2b}. Multiple regression analyses also revealed that TG concentrations were associated independently and positively with high pre-β_1-HDL, but independently and negatively with low HDL_{2b} in obese and overweight subjects. Thus, an abnormal HDL subclass distribution seems to be one of the adverse metabolic consequences of hypertriglyceridemia, as discussed by Grundy and Vega (39). Of note, the change in HDL size and distribution is already apparent at only moderately elevated TG levels.

In conclusion, our data showed the general shift toward smaller HDL particle size in obese as well as in overweight subjects. The shift was more obvious with the elevation of BMI, and especially with increased TG levels. This suggests

HDL maturation might be abnormal and RCT might be weakened in obesity.

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Antioxidant Effects of Black Rice Extract through the Induction of Superoxide Dismutase and Catalase Activities

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ABSTRACT: Our *ex vivo* study revealed that BRE had significantly stronger ability to inhibit LDL oxidation than white rice extract (WRE). The purpose of this study was to investigate whether black rice extract (BRE) supplementation might ameliorate oxidative stress and enhance antioxidant enzyme activities in HepG2 cells and in C57BL/6 mice. In the cellular study, superoxide anions ($O2^{\bullet-}$) and reactive oxygen species (ROS) in the BRE group were significantly suppressed. The BRE group also showed significant increases in superoxide dismutase (SOD) and catalase (CAT) activities by 161.6% and 73.4%, respectively. The major components responsible for the free–radical-scavenging and antioxidative properties might be cyanidin-3-O-glucoside chloride and peonidin-3-O-glucuside chloride. In the animal study, male C57BL/6 mice were divided into three groups (control, BRE, and WRE). Plasma HDL-cholesterol was significantly higher, and thiobarbituric, acid-reactive substances were significantly lower in the BRE group, whereas plasma levels of total cholesterol and triglyceride were not affected by BRE supplementation. Increased hepatic SOD and CAT activities were observed in BRE-treated mice as compared to the control mice. However, no changes were detected for the protein expression of antioxidant enzymes by Western blot analysis. Our data suggest that antioxidative effects exerted by BRE are mediated through decreases in free-radical generation as well as increases in SOD and CAT activities both *in vitro* and *in vivo*.

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Rice is a widely consumed food source for over half of the world's population (1). There are many different kinds of rice, including white rice and a variety of colored rice. Rice with colored hulls has long been considered to be a health food. Black and red rice are planted mainly in South Asian countries and other places such as the United States, Italy, and Greece. Europeans eat more black rice than South Asians (2). Recent reports have shown that supplementation of diets with black rice pigments markedly reduced atherosclerotic lesions in hypercholesterolemic rabbits and apolipoprotein–E-deficient mice (3,4). Black rice pigments are mainly located in the aleurone layer, which is characterized as dark purple to black in color and probably represents a mixture of anthocyanins. Most dietary intake of anthocyanins comes from fruits and vegetables (5). There are about 17 anthocyanins found in nature (6), whereas only six of them (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin) are ubiquitously distributed (7). The water-soluble fraction of black rice might serve as an extra source of dietary anthocyanins (8). Most people consumed a diet of rice by boiling the natural grain in water, but there are no studies concerning the effects of water extract of rice on oxidative stress. This raises the question of whether supplementation with water extract of black rice may be of importance in controlling oxidative stress.

Oxidative damage usually accompanies the development of many age-related diseases (9). Accumulating evidence suggests that oxidative modification of low-density lipoprotein (ox-LDL) may play a critical role in the development of atherosclerosis (10,11). Oxidative stress is one of the main risk factors for LDL oxidation. Reduction of LDL oxidation is an essential target that is used to evaluate dietary factors with antioxidant activity. Conjugated diene (CD) is one of the intermediate products formed during the peroxidation of polyunsaturated fatty acids in LDL lipids (12). Furthermore, thiobarbituric acid-reactive substances (TBARS) are measured as a means of indicating aldehydic breakdown products of oxidized LDL (13). In this study, we assess the effect of BRE on LDL oxidation using assays that measure CD formation *ex vivo* and TBARS levels *in vivo*.

Reactive oxygen species (ROS) are free radicals produced during metabolism and the aging process. ROS include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH). ROS can cause DNA strand breaks, base modification, lipid peroxidation, and protein modification, resulting in oxidative stress. Emerging evidence indicates that ROS are important risk factors in the pathogenesis of many diseases if the antioxidant system is impaired. The measurement of changes in endogenous antioxidant enzyme activity is considered a fairly sensitive biomarker of the response to oxidative stress. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are primary antioxidant enzymes that protect cells from damage caused by ROS. The protective mechanisms against oxidative stress by black rice extract in he-

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Abbreviations: BRE, black rice extract; C3G, cyanidin-3-O-glucoside chloride ; CAT, catalase; CD, conjugated diene; GPx, glutathione peroxidase; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; MDA, malondialdehyde; P3G, peonidin-3-O-glucuside chloride; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TG, triglycerides; TC, total cholesterol; WRE, white rice extract.

patic cells and in the animal model remain largely unknown. In this study, we investigate effects of water extracts from black and white rice on free radical generation and antioxidant enzyme activities in HepG2 cells, as well as in C57BL/6 mice. The results cast light on the physiological significance and the mechanism by which black rice suppress oxidative stress and decrease lipid peroxidation.

MATERIALS AND METHODS

Materials. The raw materials of white and black rice came from an Agricultural Research Center for the Taiwan Region, and the whole rice was washed, dried, and ground to powder. One hundred grams of rice powder was refluxed with 700 mL of double distilled water for 5 h at 80°C, cooled, and vacuum filtered through Whatman filter paper. This was repeated three times, and all extracts were pooled after lyophilization. The residue was re-dissolved in water and diluted with the reaction mixture for the subsequent assay.

Anthocyanins (cyanidin-3-O-glucoside chloride, delphinidin chloride, malvidin chloride, pelargonide chloride, peonidin-3-O-glucoside chloride, and petunidin chloride) were purchased from Extrasynthese (Genay, France) and were HPLC grade. Anti-Mn-SOD and anti-Cu,Zn-SOD antibodies were obtained from Upstate (Charlottesville, VA). Anti-catalase and anti-β-actin antibodies were obtained from Calbiochem (San Diego, CA) and Sigma Chemical Co. (St. Louis, MO), respectively.

Human LDL oxidation. Determination of CD formation was conducted in accordance with our previously reported method (14). Briefly, blood from healthy subjects was collected in the presence of 1 mM EDTA in order to prevent oxidation during lipoprotein separation. Plasma was isolated by centrifugation at 1500 × g for 15 min, and LDL was isolated by sequential density gradient ultracentrifugation (15). The isolated LDL was dialyzed against PBS (10 mM, pH 7.4) overnight and stored at 4°C before being used. Oxidation of LDL (50 μg of protein/mL) was initiated at 37°C by 5 μM $CuSO_4$ for at least 6 h. The extent of CD formation in the absence or presence of 10 μg/mL or 50 μg/mL of BRE or WRE was measured by monitoring the increase in absorbance at 234 nm as previously described (16). The protein content of the LDL fraction was determined using the Bradford assay (Bio-Rad, Hercules, CA) with BSA as the standard.

Identification of anthocyanin content in black and white rice extracts. To obtain a more comprehensive knowledge of the composition of BRE and WRE, we determined the content of anthocyanin components in BRE and WRE by HPLC and LC-MS (8). The anthocyanins were extracted using methanol/water/acetic acid (0.5:15:0.5, vol/vol/vol) according to the method described by Wu et al. (17). The extracted solutions were then diluted with acidic methanol and filtered using a 0.22-μm filter before HPLC analysis equipped with an autosampler/injector and diode array detector. An analytical SB-C_{18} column (4.6 × 250 mm, 5 μm, Agilent Technologies, Rising Sun, MD) was used for separation. The mobile phase was prepared from water/formic acid/acetonitrile (87:10:3, vol/vol/vol), eluent A, and water/formic acid/acetonitrile (40:10:50, vol/vol/vol), eluent B. The gradient program was: 5% B (5 min), 5% B to 20% B (15 min), 20% B to 40% B (15 min), 40% B to 100% B (7 min), 100% B to 5% B (3 min), 5% B (5 min). The flow-rate was 0.4 mL min^{-1}. Monitoring was performed at 520 nm, and the diode-array detector was set at an acquisition range from 200 nm to 600 nm at a spectral acquisition rate of 1.25 scan s^{-1}. To construct calibration plots, volumes of standard stock solution (1000 μM) of cyanidin-3-O-glucoside chloride, delphinidin chloride, malvidin chloride, pelargonide chloride, peonidin-3-O-glucoside chloride, and petunidin chloride were diluted with eluent A, and six concentration levels (1, 5, 20, 100, 200, 500 μM) were analyzed. For quantification, peak areas were correlated with concentrations in accordance with the calibration plot. Final concentrations were expressed as mg per 100 g of dry weight of BRE or WRE. The LC-MS was controlled by Masslynx 3.5 software (Micromass). Positive ion mode was performed with capillary voltage, 3.0 kV; cone voltage, 40V; source temperature, 120°C; desolvation temperature, 250°C; cone gas flow, 50 L/h; and desolvation gas flow, 250 L/h. Anthocyanin components were identified by both retention time and mass profile in comparison to authentic standards.

Cell culture and ROS assays. HepG2 cells were cultured in DMEM containing 100 units of penicillin/streptomycin and 10% fetal bovine serum and seeded at a density of 2×10^5 cells/well into 24-well plates. After 24-h growth at 37°C under 5% CO_2, cells were treated with BRE or WRE at a final concentration of 5 mg/mL for another 24 h. To avoid interferences due to cytotoxicity, we used a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the cytotoxic effect of BRE or WRE in HepG2 cells (18). Briefly, cultured cells were treated with BRE or WRE at a final concentration of 5 mg/mL for 24 h, and MTT solution (0.5 mg/mL) was added into the cell medium. Cells were incubated at 37°C and 5% CO_2 for an additional 4 h. DMSO and a glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) were added to each well, and the dissolved solutions were detected at 570 nm by a microplate ELISA reader (Dynex technologies, Chantilly, VA). The absorbance of untreated cells was considered as 100%. Treatment of cells with 5 μg/mL of cyanidin-3-O-glucoside chloride or peonidin-3-O-glucoside chloride was performed as the positive control group. Cultured cells were harvested in triplicate to assess the generation of $O_2^{\bullet-}$ and ROS. The ROS levels in HepG2 cells were measured using the dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (19). This reduced dye was added to cells (1×10^6 cells/mL) at a final concentration of 10 μM. The fluorescence of the oxidized dichlorofluorescein was monitored by Flow Cytometry with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Similar measurements were made with 10-μM hydroethidine (HE), which has been reported to be more specifically oxidized by $O_2^{\bullet-}$ (20). In this case, the fluorescence was monitored by Flow Cytometry with excitation at 495 nm and emission at 637 nm. The results were expressed as the relative fluorescence intensity. $O_2^{\bullet-}$ and ROS levels in HepG2 cells

without rice extract treatment were used as the negative control.

Assessment of antioxidant enzyme activities. Assays of antioxidant enzyme activities were carried out as previously described (21). Briefly, SOD activity in the extract of HepG2 or the liver homogenates of C57BL/6 mice was assayed using the hydroxylamine-reduction method (22). In this method, the reduction of hydroxylamine by $O_2^{\bullet -}$ is monitored at 550 nm, utilizing the hypoxanthine/xanthine oxidase system as the source for $O_2^{\bullet -}$. One unit (U) of SOD activity is defined as the amount of enzyme necessary to decrease the reduction of hydroxylamine by 50%. CAT activity in the extract of HepG2 or the liver homogenates of C57BL/6 mice was assayed using the method of Aebi (23). In this method, the decomposition of H_2O_2 due to CAT activity was assayed by monitoring the decrease in the absorbance of H_2O_2 at 240 nm. One U of CAT activity is defined as the amount of enzyme required to catalyze 1 μmol of H_2O_2 per min at 25°C. GPx activity in the extract of HepG2 or the liver homogenates of C57BL/6 mice was quantified by a coupled-enzyme (GPx and glutathione reductase) procedure (24). This method measures the decrease in absorbance at 340 nm as NADPH is converted to NADP. One U of GPx activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH per min. Specific activities of SOD and CAT were expressed in U/mg of protein, but GPx activity was expressed in mU/mg of protein with the protein content determined as stated above.

Animals. At eight weeks of age, 33 male C57BL/6J mice were randomly assigned to one of the three groups. Three or four animals were housed in each cage and maintained at 25°C in an animal room with a 12-h light/dark cycle. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Mice were allowed free access to food and tap water for 10 weeks during the experiment. The composition of the experimental diet is shown in Table 1. The mineral and vitamin mixtures were obtained from ICN Biochemicals (Costa Mesa, CA). To exclude that ROS generation and lipid peroxidation were affected by differences in energy utilization or hyperlipidemia, we compared body weight and plasma lipid levels of animals of the control, BRE, and WRE groups. After a 12-h overnight fast, the mice were anesthetized with CO_2, and blood samples were taken for biochemical analyses. Plasma was isolated by centrifugation at 1500 × g for 15 min and was stored at –35°C for subsequent lipid and TBARS analyses. The liver tissues were rapidly removed, blotted dry, weighed, frozen in liquid nitrogen, and then stored at –80°C for antioxidant enzyme activity analyses. Before analyses, the liver tissue was homogenized in 10 volumes of a 50-mM phosphate buffer (pH 7.4) on ice for 30 s using a polytron homogenizer. The homogenate was transferred into centrifuge tubes and centrifuged at 9000 × g at 4°C for 20 min. The supernatant was used for the measurement of protein concentration and antioxidant enzyme activities. Twenty micrograms protein was separated on SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with rabbit anti-human Mn-SOD, Cu,Zn-SOD, and catalase antibodies (dilution as recommended) using the standard Western-blot analysis. Immune complexes were visualized with horseradish peroxidase-conjugated immunoglobins and detected using enhanced chemiluminescence (Amersham Biosciences).

TABLE 1
Compositions of Normal (Control) and Experimental Diets (%)

Ingredient	Control	BRE[a]	WRE[a]
Casein	20	20	20
Corn starch	40	40	40
Sucrose	15	10	10
Rice extract	0	5	5
Soybean oil	13.5	13.5	13.5
Cellulose	5	5	5
Mineral mix	3.5	3.5	3.5
Vitamin mix	1	1	1
Choline bitartrate	0.25	0.25	0.25
tert-Butylhydroquinone	1.45	1.45	1.45
L-Cystine	0.3	0.3	0.3

[a]BRE, black rice extract; WRE, white rice extract.

Plasma lipids and TBARS analyses. The concentrations of total cholesterol (TC) and triglyceride (TG) were assayed enzymatically using commercial reagents (Merck). The levels of HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) were determined by precipitation with phosphotungstic acid/magnesium chloride and heparin/sodium citrate, respectively, using reagents supplied by Merck. The products of lipid peroxidation were measured as TBARS at 532 nm (25) using a standard curve of thiobarbituric acid (TBA) adduct formation with freshly diluted 1,1,3,3-tetraethoxypropane as the standard.

Statistical analyses. Data are given as the mean ± SD. Results were analyzed by one-way analysis of variance (ANOVA) using the SAS program (version 6.12, SAS Institute, Cary, NC). Differences between mean values were evaluated by the post-hoc test and were considered significant if $P < 0.05$.

RESULTS

Inhibition of LDL oxidation by BRE. Conjugated diene formation on exposure of copper-catalyzed LDL to 10 or 50 μg/mL rice extract is shown in Fig. 1. The initial amount of CD formed did not differ among groups. Addition of 10 and 50 μg/mL BRE significantly decreased the oxidation rates and maximal amounts of diene formation, whereas WRE had little or no effect. The lag-phase duration, which represents the resistance of LDL to lipid peroxidation, was prolonged from 72 min to 198 min when the BRE treatment was increased from 10 to 50 μg/mL. The presence of WRE at increased concentrations had no significant effect on the lag time, LDL oxidation rate, or maximal amounts of diene formation.

Quantitative analyses of anthocyanin components in rice extract. HPLC analysis was performed to estimate the content of anthocyanin components in brown and white rice extract. As shown in Table 2, cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride were identified as the two major

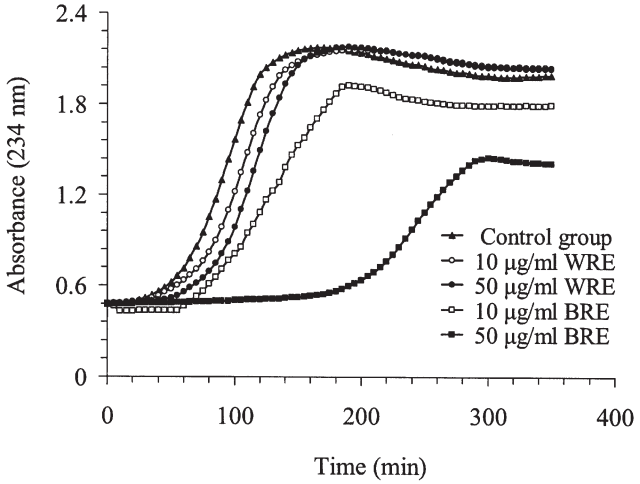

FIG. 1. Effects of white (WRE) and black (BRE) rice extracts on the generation of conjugated dienes (CD) in LDL. The oxidation of LDL (50 μg protein/mL) was initiated by the addition of 5 μM copper. White and black rice extracts were added to LDL at final concentrations as shown. CD generation was monitored by measuring the increase in absorbance at 234 nm. Values shown are from a representative experiment.

TABLE 2
Anthocyanin Content (mg/100 g dry weight) of Black Rice Extract (BRE) and White Rice Extract (WRE)

Compound	BRE	WRE
Cyanidin-3-O-glucoside chloride	26.8	14.2
Delphinidin chloride	4.3	n.d.a
Malvidin chloride	2.4	n.d.
Pelargonidin chloride	n.d.	n.d.
Peonidin-3-O-glucoside chloride	11.2	6.8
Petunidin chloiride	n.d.	n.d.

[a]n.d., not detected.

contents of BRE by comparison of the retention time and the photodiode array spectrum with those of authentic anthocyanins. We thus used cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride as the positive control to compare their effects on free-radical scavenging capacities and antioxidant enzyme activities in HepG2 cells.

Effects of rice extracts and anthocyanins on ROS levels and antioxidant enzyme activities in HepG2 cells. Cell toxicity was evaluated by MTT assay. The result from the cell viability assay showed that BRE and WRE had no obvious toxicity (94.7 ± 10.3% and 92.3 ± 6.5% of control, respectively) to HepG2 cells at the concentration of 5 mg/mL. The oxidative status was reflected from the levels of $O_2^{\bullet -}$ and ROS in HepG2 cells (Fig. 2). BRE (5mg/mL) supplementation significantly suppressed $O_2^{\bullet -}$ and ROS levels by 34.0% and 39.3%, respectively, as compared to the control group. Significant suppression of ROS generation was also obtained by cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride by 63.0% and 44.3%, respectively, at a final concentration of 5 μg/mL. However, HepG2 cells treated with a WRE (5 mg/mL) diet showed no significant difference in levels of $O_2^{\bullet -}$ and ROS compared to the control cells.

Effects of BRE, WRE, and anthocyanins on activities of antioxidant enzymes in HepG2 cells are shown in Fig. 3. SOD and CAT activities were significantly higher in the BRE group than in the control group, whereas GPx activity was not altered. However, cells treated with WRE and anthocyanins did not cause significant changes in antioxidant enzyme activities compared to the control group.

Body weight and plasma lipid levels in the C57BL/6J mice. The body weight of mice in each group before study entry (eight weeks of age) was 21.2 ± 2.6 g, and at the end of the study (18 weeks of age) was 27.5 ± 4.4 g, 25.6 ± 2.7 g, and 26.8 ± 3.3 g in the control, BRE, and WRE groups, respectively. The differences in body weight across the three groups were not significant.

No significant differences were found for the plasma levels of triglyceride, total cholesterol, and LDL-cholesterol among the BRE, WRE, and control groups. However, plasma HDL-cholesterol was significantly higher, and TBARS showed a significant decrease, in mice fed with the BRE diet, whereas no change was found in the WRE group (Table 3).

Effects of rice extracts on antioxidant enzyme activities and protein expression in livers of C57BL/6J mice. The levels of the hepatic antioxidant enzyme activities are shown in Table 4. The BRE-treated animals exhibited markedly increased hepatic SOD and CAT activities. In contrast, there was no increase in GPx activity in livers of BRE-treated animals. Hepatic SOD activity of the BRE group also displayed a greater increase than the WRE group, whereas CAT and GPx activities did not differ between the BRE and WRE groups. By using Western-blot analysis, the relatively small differences in the expression of Mn-SOD, Cu,Zn-SOD, and catalase were statistically non-significant among the BRE, WRE, and control groups (Fig. 4).

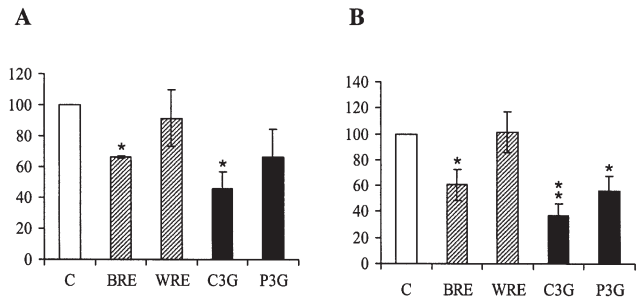

FIG. 2. Effects of rice extracts and anthocyanin components on the levels of $O_2^{\bullet -}$ (A) and ROS (B) in HepG2 cells. Cells were incubated with 5 mg/mL of rice extracts (black rice extract, BRE, and white rice extract, WRE) or 5 μg/mL of anthocyanin components (cyanidin-3-O-glucoside chloride, C3G, and peonidin-3-O-glucoside chloride, P3G) for 24 h at 37∞C. $O_2^{\bullet -}$ and ROS were determined as described in Methods. The results are representative of three different assays. All of the corresponding control values (arbitrary unit) were converted as 100%. The data represent the mean ± SD. Statistical analyses: $^*P < 0.05$ and $^{**}P < 0.01$ versus the control.

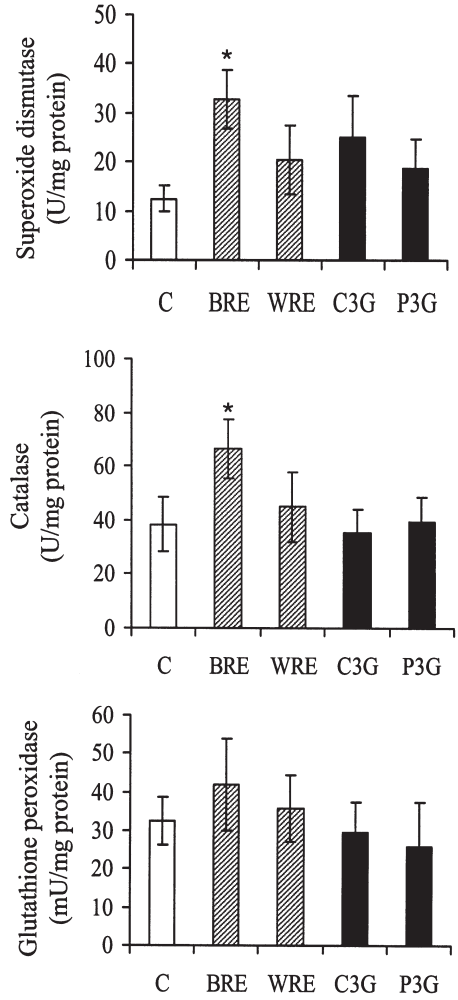

FIG. 3. Effects of rice extracts and anthocyanin components on the levels of antioxidant enzyme activities in HepG2 cells. Antioxidant enzyme activities were analyzed as described in Methods. The results are representative of three different assays. The data represent the mean ± SD. Statistical analyses: *$P < 0.05$ versus the control.

DISCUSSION

The results of this study show that black rice contains anthocyanin components with notable antioxidative properties for potential use in nutraceutical benefit in preventing human diseases pathologically related to oxidative stress (9). Cupric ion-induced LDL oxidation is a common marker for evaluating antioxidant activity relative to the prevention of lipid and protein (apolipoprotein B) oxidation (14). We have clarified for the first time that BRE evoked a remarkably greater inhibition of CD formation than that in WRE. This prompted us to investigate the antioxidant effects of BRE in cellular and animal models.

HepG2, a well–differentiated, transformed cell line, is a reliable model and widely used for biochemical and nutritional studies. In this study, we investigated the ROS scavenger effect by rice extract and anthocyanins (cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride) in HepG2 cells to assist in elucidating the mechanism of reduction in oxidative

TABLE 3
Plasma Lipid Profiles and TBARS Levels[a] in C57BL/6J Mice Fed a Control Diet with and without Black Rice Extract (BRE) or White Rice Extract (WRE

Variable	Control	BRE	WRE
n	10	12	11
TGb, mg/dL	86.7 ± 20.9	76.1 ± 22.4	94.3 ± 23.3
TCb, mg/dL	97.6 ± 15.2	102.2 ± 19.3	105.8 ± 24.2
HDL-Cb, mg/dL	58.7 ± 11.4	69.7 ± 12.0[c]	66.9 ± 14.4
LDL-Cb, mg/dL	37.5 ± 14.8	32.3 ± 08.9	35.3 ± 14.0
TBARSb, nmol MDAa/L	257.4 ± 31.5	226.7 ± 23.5[c]	243.5 ± 27.9

[a]Values are expressed as the mean ± SD.
[b]Abbreviations used: TG, triglyceride; TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde.
[c]Significant difference from the control ($P < 0.05$).

stress by BRE. Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They are widely distributed in the human diet through crops, beans, fruits, and vegetables and seem to possess strong antioxidant, anti-inflammatory, and anticarcinogenic properties (26). However, little is known about the anthocyanin profile of whole black rice. We thus quantitatively characterized the anthocyanin contents in black rice—specifically, a black rice pigmented fraction extract (BRE)—in the present study. The molecular mechanisms of the ROS-scavenging effect by BRE and anthocyanins were also determined.

The mechanism for the antioxidative effect of BRE was unclear at the start of this study. We hypothesized that BRE might exert its antioxidant effects through suppression of ROS and induction of antioxidant enzyme activities. To test this hypothesis, we determined free-radical generation and activities of SOD, CAT, and GPx in HepG2 cells treated with BRE or WRE, as well as cyanidin-3-O-glucoside chloride or peonidin-3-O-glucoside chloride. It is of note that BRE exerted its antioxidative effect via the induction of SOD and CAT activities in hepatoma cells. By contrast, enzyme activities of SOD and CAT were not affected by the addition of cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside chloride to the culture medium. Recent studies have suggested that cyanidin-3-O-glucoside chloride is thought to play an antioxidant role through the suppression of peroxynitrite-induced oxidation (8,27). Our results indicate that cyanidin-3-O-glucoside chloride and peonidin-3-O-glucoside

TABLE 4
Hepatic Antioxidant Enzyme Activities[a] in C57BL/6J Mice Fed the Control or Experimental Diets

Variable	Control	BRE[b]	WRE[b]
n	10	11	11
SOD[b] (U/mg protein)	15.6 ± 4.1	24.1 ± 06.3[c]	19.1 ± 4.8[d]
CAT[b] (U/mg protein)	40.3 ± 8.2	53.7 ± 11.4[c]	45.9 ± 9.0
GPx[b] (mU/mg protein)	32.1 ± 7.0	39.5 ± 10.2	37.8 ± 9.8

[a]Values are expressed as the mean ± SD.
[b]Abbreviations used: BRE, black rice extract; WRE, white rice extract; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.
[c]and [d]denote a significant difference from the control ($P < 0.01$) and BRE ($P < 0.05$) groups, respectively.

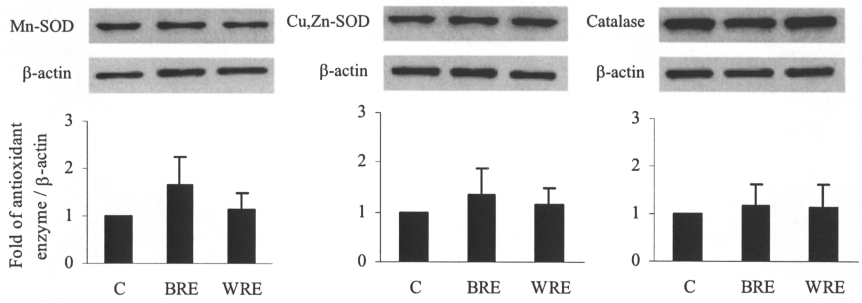

FIG. 4. Effects of black (BRE) and white (WRE) rice extracts on protein expression of Mn-superoxide dismutase (Mn-SOD), Cu,Zn-SOD, and catalase in livers of C57BL/6 mice (n = 6). Representative blots of protein expression are depicted and antioxidant enzyme expression was normalized to β-actin level as the internal control. Histograms show densitometric analyses of protein expression levels. The results are shown as fold increases above the untreated controls. Data are the mean ± SD from at least six independent experiments.

chloride may scavenge ROS through other biological pathways independent of the induction of antioxidant enzyme activities.

Antioxidant enzymes, such as SOD, CAT, and GPx, are endogenous factors that protect cells from oxidative damage caused by ROS. SOD catalyzes the dismutation of the $O2^{\bullet-}$ to molecular oxygen and H_2O_2, which in turn is metabolized to harmless water and oxygen by CAT and GPx. The activities of SOD and CAT were higher in livers of C57BL/6 mice treated with the BRE diet. These results suggest that BRE supplementation may attenuate oxidative stress by reducing ROS and increasing antioxidant enzyme activities both in vitro and in vivo. However, the increased enzyme activities observed in the BRE group may not be the result of increased gene expression via the evaluation of the protein expression of Mn-SOD, Cu,Zn-SOD, and CAT by Western-blot analysis. The lack of an exact relationship between the changes in the antioxidant enzyme activities and those in the protein levels indicates that antioxidant enzyme activities are possibly post-translationally regulated.

Previous studies have revealed that lipids are susceptible to oxidative damage. Lipid peroxidation is usually quantified by using a TBARS assay (28). Our data from plasma TBARS analyses are in accordance with the results obtained from the *ex vivo* study. Among all the major lipoproteins, HDL is considered to be an endogenous protective factor against atherosclerosis (29). Our previous studies have suggested that HDL has the potential to limit the oxidative modification of LDL (14). The results from this study suggest that BRE may also exert its antioxidative effects through the induction of plasma HDL in C57BL/6 mice.

In summary, our data shed new light on the molecular basis that BRE contributes to marked increases in SOD and CAT activities that may cause a decrease in free-radical generation and oxidative stress. This observation points to a new direction when trying to understand the physiological function of black rice as a benefit to human health.

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Phospholipid Molecular Profiles in the Seed Kernel from Different Sunflower (*Helianthus annuus*) Mutants

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ABSTRACT: Phospholipids are essential components of plant cell membranes whose acyl composition appears to be influenced by oil composition in the sunflower. In the current study, we have determined the diacylglycerol profile of the main phospholipids using phospholipase C degradation and separation of the diacylglycerols by HPLC and GLC. The main polar lipid molecular species were defined in different classes of sunflower kernel: PC, PE, and PI. The proportions of each were determined at different stages of development in order to define the point at which the mutations carried by each sunflower line affected the phospholipid composition of the seeds. The results indicated that modifications to intraplastidial *de novo* FA synthesis affected the seed phospholipid profile during the whole period of the seed formation, including accumulation and maturation, whereas the influence of mutations in the endoplasmic reticulum desaturases were more readily detected at later stages of development. These results are discussed in terms of the pathways involved in glycerolipid synthesis and phospholipid conversion in sunflower seeds.

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Phospholipids are the main components of cell membranes and therefore they are not only ubiquitous among living organisms but they are also fundamental for life (1). The galactolipids monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) are the most abundant glycerolipids in chloroplasts and plant tissues (2). However, cytosolic, mitochondrial and vacuolar membranes are composed of phospholipids, among which PC, PE, and PI are the most prominent species (3). The primary function of those phospholipids is to maintain the integrity of the cell membrane under different environmental conditions. It is also remarkable that when associated with oleosin proteins, these compounds are essential for the construction of the oil bodies present in oilseeds such as sunflower (4). These organelles accumulate the TAG synthesized in the endoplasmic reticulum, preventing their coalescence, and they anchor the lipases during the germination process. In addition to their structural function, plant phospholipids are important metabolic intermediates and indeed, PC is a substrate for the oleate desaturase (ODS) enzymes (5). The control of these metabolic pathways is tightly regulated by a series of mechanisms that depend on both the temperature and oxygen availability, and that determine the oleate/linoleate ratio of the oil accumulated in the seeds (6). On the other hand, PI and its phosphorylated derivatives are well-known intermediates in signal transduction pathways (7).

The synthesis of phospholipids in oilseeds is closely associated with TAG assembly (Fig. 1). Hence, zwitterionic phospholipids such as PE and PC are synthesized from DAG produced by the Kennedy pathway in reactions that involve the transfer of choline or ethanolamine phosphate groups. In contrast, the intermediate in PI and PG synthesis is PA, to which inositol or glycerol phosphate is added. Furthermore, phospholipids of a specific type can be converted into those of another type through different mechanisms, including the conversion of phospholipids to their DAG or PA precursors mediated by the action of C- or D-type phospholipases, or the methylation of the head residues with amine groups *via* S-adenosyl methionine (8).

Previous studies on sunflower seed kernels indicated that the most abundant phosphoglyceride species present are PC, followed by PE and PI (9). Trace amounts of the phospholipids PG and PA were also found, as well as the galactolipids MGDG and DGDG. In this regard, studies of sunflower lines carrying mutations that affect the FA composition of the oil indicate that

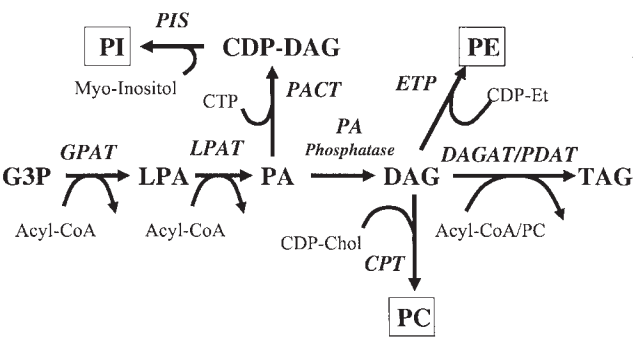

FIG. 1. General scheme of the pathway of synthesis of glycerophospholipids. G3P, glycerol-3-phosphate; LPA, lysophosphatidate; GPAT, G3P acyltransferase; LPAT, LPA acyltransferase; DAGAT, DAG acyltransferase; PDAT, PC-DAG acyltransferase; CPT, choline phosphotransferase; EPT, ethanolamine phosphotransferase; PACT, PA cytidin transferase; PIS, PI synthase

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Abbreviations: DGDG, digalactosyl diacylglycerol; MGDG, galactolipids monogalactosyl diacylglycerol; ODS, oleate desaturase; dilinoleoyl PC, PC-LL, PC-OL, oleoyl-linoleoyl PC-OO, dioleoyl-PC; PC; PDAT, phospholipid-DAG acyltransferase enzyme; PC-PL, .palmityl-linoleoyl PC; PC-PO, palmityl-oleoyl PC; PC-SL, stearoyl-linoleoyl PC.

although the absolute ratios of the seed lipid classes were not significantly altered, the FA composition of the corresponding phospholipids was also affected (9,10). As far as we can tell, these mutations do not affect the FA compositions of the leaf lipids. In other studies (11) there were no significant differences in the FA compositions of MGDG, DGDG, and PG in controls vs. those mutants analyzed. The changes in the seed acyl composition of phosphoglycerides in such lines could provide interesting information about the plant responses to the mutations responsible for their different traits. Moreover, because the 1- and 2-acyl positions within phospholipids are not equivalent, it is necessary to define the exact changes in the different molecular species of the most important sunflower seed phospholipids to obtain more complete information.

In the present study, we have defined the molecular species of the polar lipids PC, PE, and PI from different sunflower mutants at distinct stages of development. As well as the common sunflower CAS-6 line, we have studied lines that contain mutations in different genes involved in FA synthesis. The mutants suffered alterations in enzymes such as oleate desaturase, stearoyl-ACP desaturase, and β-ketoacyl-ACP synthase II that affected the acyl composition of both TAG and polar lipids. These mutations gave rise to the high-oleate CAS-9 line, the high-stearate high-linoleate CAS-3 line, and the high-palmitate high-oleate CAS-12 line (12–14). The study of the polar lipids provided information about the impact of these mutations on functional membrane lipids, as well as on the ability of the plant to compensate for their excess of saturated FA. Finally, we discus the mechanisms of phospholipid synthesis and conversion in sunflower seeds in relation to the results obtained.

EXPERIMENTAL PROCEDURES

Plant material. Sunflower seeds were germinated in wet perlite at 25°C and transferred to a germination chamber for 2 wk for full seedling development. Subsequently, they were moved to growth chambers equipped with fertigation lines and maintained at 25°/15°C (day/night), with a 16-h photoperiod and a photon flux density of 300 $\mu mole \cdot m^{-2} \cdot s^{-1}$ until harvested.

Lipid extraction. Total lipids were extracted following the method described by Hara and Radin (15). Freshly harvested sunflower kernel endosperm weighing from 0.05 to 0.5 g was ground in 5 mL of hexane/2-propanol 3:2 in a screw-capped tube using a glass bar and sea sand until the tissue was reduced to a fine powder. The resulting suspension was heated at 80°C for 10 min before adding 2.5 mL of sodium sulfate 6.7% and vigorously shaking the tubes. The hexane-enriched supernatant was separated by centrifugation at 5,000 × *g* for 5 min. in a benchtop centrifuge and transferred to a clean tube. The aqueous residue was again extracted with 3.75 mL of hexane/2-propanol 7:2, and the resulting organic phases were combined with those previously obtained. The total lipid extract was then submitted to fractionation.

Lipid fractionation. Crude lipid extracts obtained in the preceding step were evaporated under nitrogen and the residues dissolved in 5 mL of chloroform. The resulting solutions were fractionated following the protocol described by Nash and Frankel (16) in Lichrolut 0.5 g silica gel cartridges (Merck, Darmstadt, Germany) that were set in a vacuum manifold and equilibrated with 2 mL of chloroform. Total lipid extracts were loaded and washed with another 15 mL of chloroform to elute the neutral lipids from the column, and with 10 mL of methanol to quantitatively recover polar lipids. This fraction was evaporated to dryness under nitrogen, dissolved in 0.15 mL of chloroform/methanol (2:1) and the lipid classes were separated by TLC.

TLC fractionation. Purified lipid fractions were loaded onto TLC plates (Si60, 5 μm, Merck, Darmstadt, Germany) and developed with chloroform/methanol/acetic acid/water (85:15:5:2.5). Bands were visualized by spraying the plates with a solution of 2,3-dichlorofluoresceine in methanol, and they were identified by comparing their Rfs with those of the corresponding standards. Bands corresponding to PC, PE, and PI were scrapped from the plate and the lipids were eluted from the silica gel with 10 volumes of chloroform/methanol (2:1).

Derivation of phospholipids by phospholipolysis. The polar lipids purified by TLC were subjected to phospholipase C hydrolysis to form their corresponding DAG (17). Phospholipid solutions containing approx. 1 mg of the corresponding lipid were evaporated to dryness under nitrogen and resuspended in 1 mL of 50 mM potassium phosphate pH 7.2, 2 mM $CaCl_2$. The suspension was fully dispersed by applying several pulses of sonication with a probe sonicator. For PC, a volume of enzyme stock corresponding to 5 U of phospholipase C from *Chlostridium perfringes* was added and the reaction mixture was incubated at 30°C for 1 h. In contrast, phospholipase C from *Bacillus cereus* was used for PE and PI, the former requiring 5 U of the enzyme in each assay while 10 units were added for PI. The reaction mixtures were incubated at 30°C for 1 and 3 h, respectively, to complete hydrolysis. Reactions were stopped by the addition of 0.1 mL 6 M HCl and 1 mL sodium sulfate 6.7%. The DAG were extracted three times with 2 mL hexane, whereafter the organic phases were pooled, evaporated under nitrogen, dissolved in acetonitrile/acetone (70:30), and subjected to HPLC and GLC analysis.

HPLC analysis. DAG molecular species obtained from hydrolysis of PC by phospholipase C were analyzed by reverse phase HPLC in a C18 column (Ultrasphere ODS 4.6 × 250, Beckman, Fullerton, CA). Samples were dissolved in equilibration solvent and injected into the column equilibrated with acetonitrile/acetone (70:30). The different molecular species were then eluted by increasing the acetone content to a final ratio of acetonitrile/acetone of 50:50 after 24 min. Then polarity was increased in one minute to 15:85 acetonitrile/acetone, and that composition was maintained for 4 min. Subsequently, the column was equilibrated with the initial solvent mixture in preparation for the next injection. The molecular species were identified by either comparing their retention times with those of available commercial standards. Some peaks were identified by determining their FA composition by GLC.

This HPLC method allowed the separation and quantification of all the molecular species present in sunflower seed phos-

pholipids in the form of DAG, except for the pair 1-palmitoyl-2-oleoyl glycerol/1-stearoyl-2-linoleoyl glycerol that had to be resolved by GLC. The PC isolated from the seed kernels was easily degraded by phospholipase C from *Clostridium perfringens*. However, because this enzyme was not active on PE or PI, these phospholipids were degraded with phospholipase C obtained from *Bacillus cereus*. Although the yield of the degradation was complete and quantitative for PC and PE, PI was only partially degraded (approx. 50%). Determinations were carried out using three different populations of seeds coming from different plants.

HPLC system. HPLC separation was carried out in a Waters 2695 Module (Milford, MA) equipped with a Waters 2420 ELS detector. The flow rate was 1 mL/min, and samples were dissolved in the same solvent in which the column was equilibrated at the time of injection. Data were processed using Empower software. Fraction collection was carried out with a Waters on-line fraction collector III. The evaporative light scattering detector was regularly calibrated using high purity commercial standards of each lipid.

Analysis of DAG by GLC. The GLC method used was a modification of that described by Perez-Camino *et al.* (18). DAG produced from the different phospholipid classes were evaporated to dryness under a flowing nitrogen. Then, 50 µL of a silanizing mixture (1 mL tetramethylsilane, 3 mL hexadecyl disilazane, 5 mL dry pyridine) per mg of sample were added, and the reaction was left to stand at room temperature for 15 min. The resulting TMS-DAG were dissolved in 0.2 mL heptane and analyzed in an Agilent 6890 gas chromatograph (Palo Alto, CA), using a DB-17HT column (25 m length, 0.25 mm internal diameter, 0.15 µm phase thickness; J&W Scientific, Folsom, CA) and hydrogen as the carrier gas. The injector and FID detector temperature was 340°C, whereas the column temperature was set at 240°C. Mass spectrometry of TMS-DAG species was performed with a Finnigan MAT95 high-resolution mass spectrometer (Finnigan, Bremen, Germany) interfaced with a Hewlett–Packard 5890 Series II gas chromatograph (Palo Alto, CA) using the aforementioned conditions for chromatography. Electron impact (EI) spectra were recorded at 70 eV and full spectra (50–1000 amu) with a scan speed of 2 s decay^{-1} over the entire elution profile. Data were analyzed using an ICIS II Data System from Finnigan MAT (Finnigan, Bremen, Germany).

Analysis of FAME. FAME were prepared by treating the lipid sample with 3 mL of methanol/toluene/sulfuric acid (88:10:2, by vol) for 1 h at 80°C (19). After cooling, FAME were extracted twice with 2 mL of heptane and analyzed by GLC using a Supelco (Bellefonte, PA) SP-2380 fused silica capillary column (30 m length, 0.25 mm i.d., 0.20 µm film thickness). Hydrogen was used as the carrier gas at 28 cm·s^{-1}, the detector and injector temperature was 200°C, and the oven temperature was 170°C. Different methyl esters were identified by comparison with known standards.

RESULTS AND DISCUSSION

Sunflower seeds accumulate carbon and energy in the form of TAG in order to feed the embryo during germination. Seed genesis and oil storage is a process that is carefully regulated, and it typically lasts from 40 to 45 d in commercial *Helianthus*. As reported for other oil crops (20), three different phases can be distinguished within this period. The first of these involves cell division and little accumulation of reserves, and it extends from 0 to 10–15 d after flowering (daf). A rapid deposition of oil then takes place in a second phase that embraces from 15 to 30 daf, this being the period in which *de novo* FA and TAG synthesis is most active. Finally, the last stage of seed development involves desiccation and the shutting down of the metabolic pathways involved in oil synthesis. Therefore, we studied the molecular species of the most prominent membrane lipid classes present in sunflower at each of these three phases, denominated stage 1 (15 daf), stage 2 (25 daf), and stage 3 (40 daf). Sunflower mutants with modified seed FA composition provide us with a good model to study how these changes in FA affect membrane phospholipids during seed development and desiccation. The FA compositions of the mutant seeds used in this work are shown in Table 1.

PC molecular species. The most abundant phospholipid class in the sunflower was PC (9). This phospholipid has both important structural and metabolic roles in plant physiology. Not only is it the main nonphotosynthetic membrane component, but it is also the substrate for oleate desaturation in the endoplasmic reticulum by the action of the ODS isoenzymes (5). Thus, the linoleate present in oilseeds is produced by desaturating the oleate present in the *sn*-2 position of PC. Hence, if we take into account the high proportion of TAG when compared to the PC content in oil seeds (approx 90:1), we can conclude that there is a considerable exchange of acyl moieties between these two lipid classes via DAG, lysophosphatidate intermediates (21), or through the direct action of the phospholipid-DAG

TABLE 1
Total Lipid FA Composition of Different Sunflower Lines

	FA (mol%)							
Line	16:0	16:1	18:0	18:1	18:1$^{\Delta 11}$	18:2	20:0	22:0
CAS-6	4.9	—[a]	6.5	36.8	—	50.9	0.3	0.6
CAS-9	3.9	—	5.6	87.4	—	1.7	0.4	0.9
CAS-3	5.7	—	24.5	13.0	—	54.3	1.0	1.5
CAS-12	31.0	4.1	1.7	46.4	2.2	12.5	1.0	1.1

[a]— = <0.5%

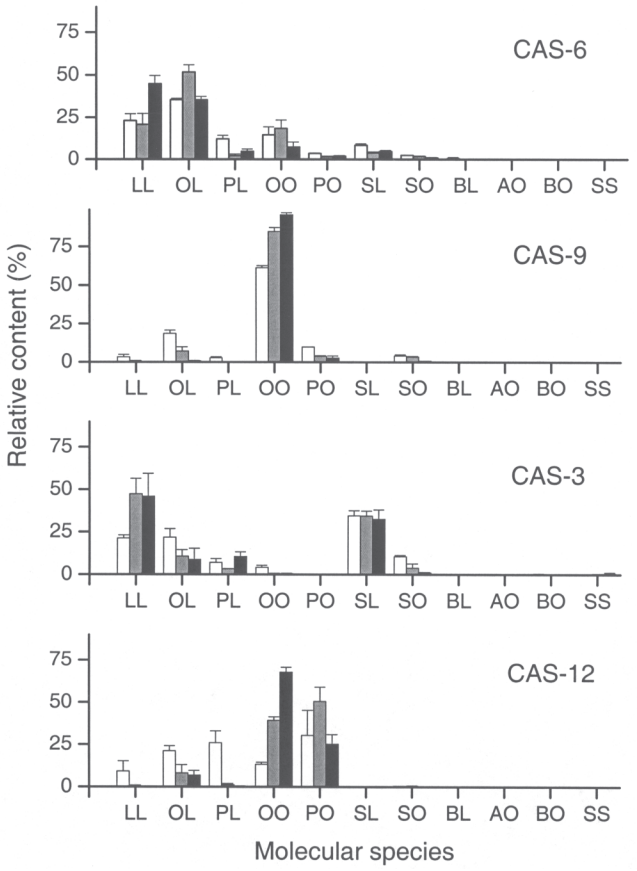

FIG. 2. Molecular species corresponding to PC isolated from common sunflower (CAS-6), high-oleic (CAS-9), high-stearic high-linoleic (CAS-3), and high-palmitic high-oleic (CAS-12). Data are the averages of three independent determinations ± SD. Lipids were isolated from sunflower kernels at 15 (stage 1, □), 25 (stage 2, ▒), and 40 (stage 3, ■) days after flowering. The molecular species were coded with two letters representing the FA. Palmitic acid (P), stearic acid (S), oleic acid (O), linoleic acid (L), arachidic acid (A), and behenic acid (B) were present in the molecular species quantified.

acyltransferase enzyme (PDAT; 22). The close relationship between TAG and PC metabolism was reflected in the different profiles of the accumulation of PC species between sunflower mutants (Fig. 2).

In the case of common sunflower lines, stages 1 and 2 were characterized by a higher content of species containing oleate and linoleate, mainly oleoyl-linoleoyl PC (PC-OL), dilinoleoyl PC (PC-LL), and some dioleoyl-PC (PC-OO). These species increased at stage 3 when the main species found were PC-LL and PC-OL. This molecular profile of PC species indicated that the synthesis of oleate predominates at the beginning of oil accumulation but the action of endoplamic reticulum ODS later shifts the emphasis toward linoleate accumulation, in agreement with previous studies on TAG accumulation (23). The activation of ODS takes place during stage 2 of development, and it stays active until the seed is fully developed and it enters the drying period. This is reflected by the increases in the PC linoleate containing species from stage 2 to stage 3. Furthermore, species such as palmityl-linoleoyl PC (PC-PL), palmityl-oleoyl PC (PC-PO) and stearoyl-linoleoyl PC (PC-SL) were relatively more abundant in stage 1 than stage 2, when they diminished before increasing again in stage 3. This may be caused by the high rates of oleate synthesis characteristic of the exponential phases of oil accumulation. Indeed, this is when the plastidial condensation enzymes, the stearoyl-ACP desaturase, and acyl-ACP thioesterase A enzymes display the highest levels of activity (24).

The profile of PC species found in the high-oleate CAS-9 mutant was, on the other hand, very different from that in the common sunflower. This line is characterized by a mutation that silences the endoplamic reticulum ODS during seed development (25). For this reason the PC-OO species was always the most abundant at each of the three stages studied. Indeed, the relative abundance of this species did alter during development, accounting for 60% of the total PC during stage 1 and increasing to 90 and 96.5% during stages 2 and 3, respectively. However, the presence of PC species containing relatively high proportions of linoleate PC-LL and PC-OL during stage 1 indicated that there was probably an ODS that was active in this period that was later turned off, in agreement with earlier results (10). Moreover, the considerable accumulation of oleate in the PC species was remarkable in this mutant. Thus, PC-OO represented more than 95% of the total PC fraction, representing the highest oleate content of the lipid species found in this plant. This could be due to the accumulation of an oleate acyl moiety at the *sn*-2 position of PC where it would normally be desaturated by ODS. Because the rate of desaturation diminished in high-oleate mutants, this FA remained in PC during the period of seed kernel development.

A different profile of PC species appeared in the high-stearate line CAS-3 in which the high-stearate trait was maximally expressed in PC during the first stage of development. During this period, the PC-SL species accounted for more than 30% of the total PC, and this was maintained throughout stages 2 and 3. The early accumulation of stearate was probably due to the early intraplastidial modification of lipid synthesis that occurs in this mutant. Thus, CAS-3 is a SAD-deficient mutant (13) that expressed its high-stearate phenotype throughout the whole period of seed development (23), altering both *de novo* FA synthesis and glycerolipid synthesis. In this mutant, the accumulation of stearate in PC was coupled to an increase in the accumulation of linoleate-containing species such as PC-LL and PC-OL in the seeds, the former to a similar level as that in the common CAS-6 line. This was in agreement with earlier observations of high linoleate content in the oil of highly saturated sunflower lines. Indeed, this accumulation of linoleate indicates that the excess of stearate in the membranes is compensated for by a mechanism that activates ODS enzymes, increasing the content of PUFA to maintain membrane fluidity.

PC species isolated from the high-palmitate high-oleate mutant CAS-12 accumulated early, like the saturated FA in membrane lipids. Thus, PC-PL and PC-PO were the most abundant species at 15 daf, with a relatively high proportion of species containing oleate and linoleate. Again, mutation of a gene encoding for an intraplastidial enzyme such as β-ketoacyl-ACP

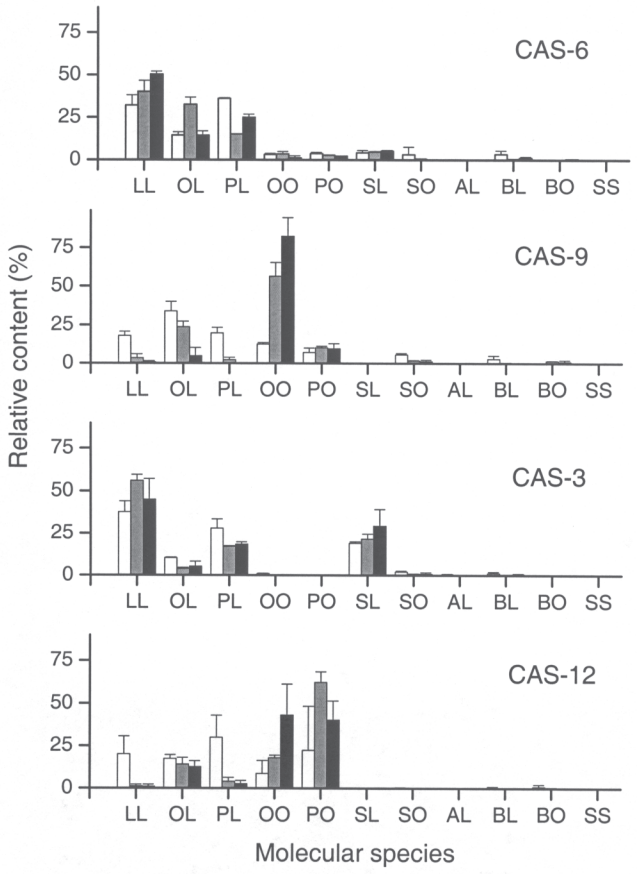

FIG. 3. Molecular species corresponding to PE isolated from common sunflower (CAS-6), high-oleic (CAS-9), high-stearic high-linoleic (CAS-3), and high-palmitic high-oleic (CAS-12). Data are the average of three independent determinations ± SD. Lipids were isolated from sunflower kernel at 15 (stage 1, □), 25 (stage 2, ■), and 40 (stage 3, ■) days after flowering. The molecular species were coded with two letters representing the fatty acids. Palmitic acid (P), stearic acid (S), oleic acid (O), linoleic acid (L), arachidic acid (A), and behenic acid (B) were present in the molecular species quantified.

synthase II induced the early expression of the trait (23). This was associated with a concomitant increase of the ODS enzymes in stage 1, probably to compensate for the excess of palmitate in the membranes. Subsequently, in stages 2 and 3 oleate accumulated in PC due to the lack of endoplasmic reticulum ODS expression. Hence, the main species in the PC fraction of the CAS-12 mutant were PC-OO and PC-PO. An unexpectedly high content of PC-OO was again found in this mutant, indicating that this is a common occurrence in high-oleate sunflower mutants.

PE molecular species. Like PC, PE was also modified by mutations that altered the FA composition of the oil in the different sunflower mutants. The variation observed in the PE species in the common sunflower line CAS-6 was similar to that found for PC. Hence, there was an accumulation of dilinoleoyl PE (PE-LL) at all three stages, whereas the accumulation of oleoyl-linoleoyl PE (PE-OL) reached a maximum at stage 2. This PE species later decreased, leading to an increase the content of PE-LL in mature seeds probably due to the action of endoplamic reticulum ODS (Fig. 3). It was also remarkable that more PE species containing saturated FA were detected than for PC. These phospholipids containing saturated FA accounted for 33% of the total PE content in the fully developed kernels, more than double the 15% of PC species with saturated FA. Indeed, palmitate was more abundant in PE at stages 1 and 3 of development, which is probably related to the synthesis of cell membranes and those of the oil bodies. The high-oleate line CAS-9 also displayed an enrichment of oleate in the PE species. Unlike PC, this trait was not evident in seeds harvested 15 daf, when the predominant species were PE-OL and palmitoyl-linoleoyl PE. Rather, the high-oleate trait was evident in stages 2 and 3, when dioleoyl PE (PE-OO) was the most abundant molecular species, followed by PE-OL and palmitoyl-oleoyl PE (PE-PO). The relative abundance of PE-OO in mature seeds was lower than that in PC at the same stage, which probably reflected a lower accumulation of the oleate acyl moieties in PE. The phenotype of the high-stearate CAS-3 mutant was again expressed at early stages of development, with a predominance of PE-LL and stearoyl-linoleoyl PE at the three stages studied. Again, PE species were enriched in palmitate when compared with those found in PC phospholipids, as seen in the control and high-oleate lines. Finally, the high-palmitate high-oleate CAS-12 line displayed the mutant phenotype in PE at an earlier stage, with a higher relative abundance of PE-PO, again reflecting the preference of PE to accumulate palmitate. The proportion of both PE-PO and PE-OO species increased at stages 2 and 3, at the expenses of species containing linoleate.

PI molecular species. The third lipid class studied here was PI, present in sunflower kernels at similar levels as PE. However, PI fulfills different functions and these phospholipids are synthesized through different pathways. Thus, they displayed a very different composition, with a higher proportion of saturated FA, as reported previously (10). Although PI is synthesized from phosphatidate and does not therefore serve as a direct TAG precursor (Fig. 1), the acyl composition of the oil has an important impact on the molecular PI species. These changes were most apparent at stages 2 and 3, when oil was accumulating more actively. At stage 1, all mutants displayed similar PI profiles, in which palmitoyl-linoleoyl PI (PI-PL) was most abundant (Fig. 4). Nevertheless, the early accumulation of stearate and linoleate in CAS-3 made stearoyl-linoleoyl PI (PI-SL) almost as abundant as the PI-PL species at the beginning of seed development. Furthermore, the CAS-6 common sunflower line accumulated PI-PL and PI-SL as the main species at later stages of development. The predominant species found at stages 2 and 3 in CAS-3 and CAS-12 were PI-SL and palmitoyl-oleoyl PI respectively, whereas concomitantly a high-dioleoyl PI content was displayed by the CAS-9 line. This saturated FA-rich composition indicated that enzymes involved in PI synthesis are more specific for glycerolipids carrying these acyl moieties. Hence, the enzyme PI synthase (26) seemed to be the number one candidate to select these species. Indeed, it has been indicated that PI synthase from the organs of different plants shows a high selectivity for

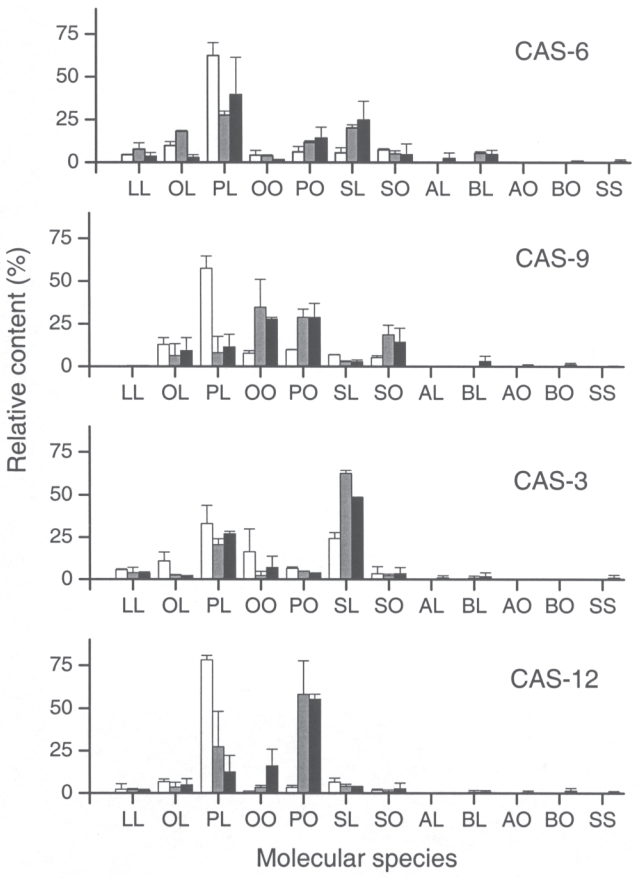

FIG. 4. Molecular species corresponding to PI isolated from common sunflower (CAS-6), high-oleic (CAS-9), high-stearic high-linoleic (CAS-3), and high-palmitic high-oleic (CAS-12). Data are the average of three independent determinations ± SD. Lipids were isolated from sunflower kernel at 15 (stage 1, □), 25 (stage 2, ■), and 40 (stage 3, ■) days after flowering. The molecular species were coded with two letters representing the FA. Palmitic acid (P), stearic acid (S), oleic acid (O), linoleic acid (L), arachidic acid (A), and behenic acid (B) were present in the molecular species quantified.

the synthesis of saturated-unsaturated PI species (27). Moreover, similar results were observed in maize coleoptile microsomes (28) and in *E. coli* expressing an exogenous PI synthase from Arabidopsis (29). The importance and function of this enrichment in saturated FA of PI has not been yet determined.

The results obtained here indicated that mutations affecting the FA composition of sunflower oil have a direct impact on the accumulation of different molecular species of membrane phospholipids. Furthermore, the acyl composition of phospholipids appears to be dependent on the stage of seed development. These changes were mainly related to the activity of the enzyme ODS and the intraplastidal *de novo* FA biosynthetic pathway. The relatively early accumulation of PC-LL and PE-LL species in the high-stearate CAS-3 mutant indicated that there are mechanisms that activate ODS when saturated FA increase in cell membranes. However, it is not clear how the plant might detect this situation or how this might produce such a response. It was also remarkable that PC contained an unexpectedly high proportion of diolein moieties in the high-oleate mutants CAS-9 and CAS-12. This probably reflects the high accumulation of oleate in the *sn*-2 position of PC as well as the high proportion of oleate in the acyl-CoA pool that would thus be easily incorporated in the *sn*-1 and *sn*-2 positions by the endoplasmic reticulum glycerol-3-phosphate acyl-CoA acyltransferase and lysophosphatidate acyl-CoA acyltransferase, respectively. Nevertheless, the molecular species of PE and PC were very similar in all the sunflower lines studied indicating that they share an important part of their biosynthetic pathways. In contrast, the acyl composition of PI was different displaying higher saturated FA content, stearate or palmitate depending on the mutant. This also revealed the selectivity of the enzymatic machinery involved in the synthesis of this phospholipid.

The results regarding PC species gave interesting information about how TAG are assembled in the sunflower, indicating that PC and TAG share DAG as their direct precursor. Furthermore, the different molecular species provide data about the specificity of some of the enzymes in the Kennedy pathway, which have been shown to be an important constraint at the time of isolating sunflower mutants with a higher saturated fatty acid content. This aspect of TAG biosynthesis could be examined by comparing the composition of the PC and TAG species in each mutant. Thus, in the CAS-6 common sunflower the predominant species among the TAG were dioleoyl-linoleoyl glycerol, oleoyl-dilinoleoyl glycerol, and trilinoleoyl glycerol. This indicates that TAG were mostly assembled from the most abundant PC (or DAG) species containing oleoyl-linoleoyl and dilinoleoyl acyl moieties (Fig. 2), and the oleoyl and linoleoyl-CoA substrates. On the other hand, the TAG in the high oleic lines were comprised of approximately 70% trioleoyl glycerol, followed by 12% of stearoyl-dioleoyl glycerol (30). This proportion contrasted with the compositions of more than 95% PC-OO and less than 1% of stearoyl-oleoyl PC, indicating that most stearic acid was transferred to glycerolipids via acyl-CoA-diacylglycerol acyltransferase or PDAT in the last step of the pathway. This hypothesis was supported by the TAG and PC composition of the high stearic line CAS-3, in which the predominant TAG were distearoyl-linoleoyl glycerol, stearoyl-oleoyl-linoleoyl, and stearoyl-dilinoleoyl glycerols, produced by esterification of stearoyl-CoA on dilinoleoyl, stearoyl-linoleoyl, or oleoyl-linoleoyl DAG or PC precursors. Thus, by considering the molecular species of PC in sunflower, it appears that the first enzyme of the Kennedy pathway, GPAT has low specificity for stearoyl-CoA and a preferance for linoleate, oleate or palmitate derivatives. The following condensing enzyme, LPAAT was very specific for oleoyl-CoA which was quickly desaturated to linoleate in the *sn*-2 position of PC, whereas stearate is preferentially introduced at the *sn*-3 position by the DAGAT or PDAT enzymes. These aspects of sunflower lipid biosynthesis have been confirmed by biochemical studies and studies of the FA distribution in the TAG molecules of sunflower. These studies indicated that stearic acid was asymmetrically distributed in sunflower TAG (31), an important constraint at the time of producing sunflower oils with very high content of this FA.

Summarizing, this work shows that modifications in storage

TAG from sunflower seeds have a direct effect on membrane lipids, provided they share biosynthetic pathways. This means that modifications in the FA biosynthesis introduced in new lines of sunflower or other oil crops are limited by the impact of these modifications on the functionality of the seed membranes. Furthermore, the study of the composition of molecular species of phospholipids revealed interesting aspects of TAG biosynthesis in sunflower, like the sequential action of the enzyme ODS and the specificity of the acyltransferases of the Kennedy pathway.

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Comparative Study of Lipids in Mature Seeds of Six *Cordia* Species (Family Boraginaceae) Collected in Different Regions of Brazil

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ABSTRACT: The oil content, FA, and lipid class composition of the mature seeds of six *Cordia* species were analyzed. Mature seeds of each species were collected in their natural habitat from 2002 to 2004. The total lipid content varied from 1.9% to 13.2%, there being significant differences between the results found in different years for each species and between the species analyzed. The contents of FFA varied from 2.0% to 7.9% of total lipids. Neutral lipids (NL) were the largest class, making up between 89.6% and 96.4% of the total lipids; the phospholipids (PL) were the second largest class (3.0% to 8.9% of the total lipids), and the glycolipids (GL) were the smallest class (0.6 to 3.4%). The presence of GLA was determined in each class of lipids; it is predominant in the NL. Levels of GLA ranged from 1.2% to 6.8% of total seed FA. This is, to our knowledge the first study of lipid composition in seeds of species of *Cordia* from Brazil.

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GLA, a FA belonging to the omega-6 (ω-6) family, has received considerable attention in recent years due to its beneficial effects on the treatment and control of: cardiovascular disease (1), diabetes (2), atopic dermatitis (3), alcoholism (4), premenstrual syndrome (5), hypertension (6,7), and tumors (8). Studies have also shown that it reduces VLDL, LDL, and cholesterol levels (9,10).

Heath products containing GLA mainly consist of lipid extracts from *Oenothera biennis* L., *Borago officinalis* L. and *Ribes nigrum* L. (11). But other species belonging to the Boraginaceae, Aceraceae, and Ranunculaceae families can also be potential sources of GLA. A study of 36 species, belonging to 20 genera of the Boraginaceae family showed that all the accessions analyzed contained GLA and that GLA was a potential chemotaxonomic marker for this family (12). Although no species of *Cordia* (Boraginaceae) were included in that study, the results suggested that other Boraginaceae species might also contain GLA. Although previous studies reported the FA composition of the seed oil of several wild and cultivated *Cordia* species (13–18), they did not included Brazilian *Cordia* species. In a study of three Brazilian *Cordia* species (*C. ecalyculata, C. myxa,* and *C. selowiana)*, the analysis of the FA composition of seed oil revealed the presence of GLA (11). For this reason, we found it interesting to further evaluate Brazilian *Cordia species* for GLA content.

The objective of this study was to analyze the composition of lipids in mature seeds of six different native Brazilian species of *Cordia,* with emphasis on determining the presence of GLA, and to see whether this acid is found in the reserve lipids.

EXPERIMENTAL PROCEDURES

Sample collection and preparation. Seeds from mature plants of *Cordia* species were collected in different regions of Brazil (Table 1). The species used were *Cordia ecalyculata, C. glabrata, C. sellowiana, C. superba, C. trichotoma,* and *C. goeldiana.* The seeds were peeled, and only the nut and mesocarp were ground and homogenized in a food processor. The obtained powder was immediately analyzed. Three samples were taken from each plant at each location and time of collection, and these replicates were processed individually.

Extraction and quantification of total lipids. Twenty grams of the homogenized powder obtained from the procedure just described were extracted according to the method of Bligh and Dyer (19). Samples were first extracted with 50 mL of chloroform/methanol/water (1:2:0.8, by vol), and then with 50 mL of the same solvent system but with a different proportion (2:2:1.8). The extract was filtered and dried using sodium anhydride, and 10 mL of this extract was transferred to a previously weighed beaker and kept in an oven at a temperature of 105°C until constant weight. Total oil content was measured gravimetrically and expressed as a percentage of dry seed weight.

Separation of different classes of lipids by column chromatography. Lipids were separated following the procedure described by Christie (20). Total lipids were partitioned into classes by column chromatography. A glass column (20 cm long by 1.25 cm i.d.) containing 20 g of silica gel 60 (70–230 mesh, Merck) was used as stationary phase, and chloroform, acetone, and methanol were sequentially applied to the column

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TABLE 1
Place and Date of Collection, Total Lipid Content, and Fractions (g/kg) of the *Cordia* L. Seeds Analyzed[a]

Species	Place of collection[b]	Date of collection	Total lipid content (%)	FFA content (%)	Major lipid classes (% of total)		
					NL	GL	PL
C. *ecalyculata* Vell.	(1)	April 2002	9.7 ± 0.4[a]	2.8 ± 0.2[a,c]	95.0 ± 1.8	1.0 ± 0.6	4.0 ± 1.2
		April 2003	7.3 ± 0.4[b]	3.2 ± 0.3[a,g]	94.1 ± 1.5	0.9 ± 0.4	5.0 ± 0.8
		April 2004	8.4 ± 0.5[c]	3.3 ± 0.2[a,g]	94.7 ± 1.3	1.1 ± 0.8	4.2 ± 1.0
C. *glabrata* DC.	(2)	October 2002	2.6 ± 0.4[d]	7.3 ± 0.2[b,f]	92.3 ± 1.1	1.9 ± 0.8	5.8 ± 1.1
		October 2003	1.9 ± 0.8[d]	7.7 ± 0.4[b]	91.0 ± 1.6	3.2 ± 0.7	5.8 ± 1.2
		October 2004	2.1 ± 0.1[d]	7.4 ± 0.8[b]	91.1 ± 1.8	3.4 ± 1.0	5.5 ± 0.8
C. *goeldiana* Huber	(3)	December 2002	2.2 ± 0.1[d]	7.9 ± 0.7[b]	89.8 ± 1.4	2.8 ± 0.8	7.4 ± 0.8
		November 2003	2.5 ± 0.3[d]	7.6 ± 0.3[b]	90.7 ± 1.7	3.1 ± 1.0	6.2 ± 0.9
		December 2004	2.3 ± 0.2[d]	7.8 ± 0.2[b]	89.6 ± 1.6	3.4 ± 0.5	7.0 ± 0.9
C. *sellowiana* Cham.	(4)	October 2002	13.2 ± 0.7[e]	2.0 ± 0.2[c]	90.6 ± 1.8	2.5 ± 0.8	6.9 ± 1.2
		September 2003	9.5 ± 0.3[a,c]	2.3 ± 0.2[a,c]	91.0 ± 1.7	1.9 ± 0.6	7.1 ± 0.9
		October 2004	9.3 ± 0.3[a,c]	2.5 ± 0.6[a,c]	91.8 ± 1.9	2.6 ± 0.6	5.6 ± 1.0
C. *superba* Cham.	(5)	September 2002	6.0 ± 0.4f	4.6 ± 0.4[d,h,i]	89.7 ± 1.5	3.2 ± 0.9	7.1 ± 1.0
		September 2003	5.3 ± 0.5[f,g]	4.5 ± 0.6[d,i]	89.5 ± 1.7	2.9 ± 1.0	7.6 ± 0.9
		October 2004	4.4 ± 0.4[g,h]	4.1 ± 0.5[d,g]	89.7 ± 1.5	1.4 ± 0.6	8.9 ± 1.2
C. *trichotoma* Vell.	(4)	June 2002	4.3 ± 0.3[g,h]	5.4 ± 0.4[i,e]	96.4 ± 1.7	0.6 ± 0.5	3.0 ± 0.8
		June 2003	3.1 ± 0.2[d,i]	5.6 ± 0.2[e,h]	94.2 ± 1.9	1.8 ± 0.9	4.0 ± 1.0
		June 2004	3.9 ± 0.3[h,i]	6.3 ± 0.6[e,f]	94.6 ± 0.9	2.3 ± 1.0	3.1 ± 0.8

[a]Results are expressed as means ± SD of three measurements. Means within a column that do not share a superscript letter are significantly different ($P \leq 0.05$).
[b](1) São Paulo state (city of Rio Claro); (2) Mato Grosso state (Xingu Indian Reservation); (3) Pará state (city of Boa Vista); (4) São Paulo state (city of Vargem); (5) Minas Gerais state (city of Bueno Brandão).

to elute neutral lipids (NL), glycolipids (GL), and phospholipids (PL), respectively. The lipid fractions were reduced in volume at 40°C using a rotary evaporator. Then 100–300 mg of each fraction were transferred to preweighed flasks; the residual solvent was evaporated under nitrogen, and the samples were placed in a desiccator until constant weight. The content of each lipid class was calculated by weight, and expressed as a percentage of the total lipid dry weight.

FFA content. The FFA content of the lipid fractions was determined colorimetrically according to Kwon and Rhee (21). The total content of FFA in the samples was expressed as oleic acid equivalents using a standard curve.

GLC of FAME. Total lipids were converted into methyl esters of FA using BF_3 methanol as esterifying agent, according to the method suggested by the American Oil Chemist's Society (22). The methyl esters were diluted in hexane and analyzed by GC using a Chrompak chromatograph (model CP 9001) with a flame ionization detector and a CP-Sil 88 capillary column (Chrompak, WCOT Fused Silica 59 m × 0.25 mm). Detector temperature was 270°C, and temperature of the injector was 250°C. Initial temperature was 80°C for 7 min, programmed to increase 10°C per min up to 180°C, hold for 3 min, and then increase 3°C/min up to a maximum temperature of 210°C. Carrier gas used was hydrogen at a flow rate of 2 mL/min. The identification of the FA was done by comparison of the retention times of the sample components with authentic standards of FAME injected under the same conditions. Coelution (spiking) of samples was also used. FA composition, as percentage of total FA weight, was calculated using area counts of the chromatogram.

GC–MS. The FA composition was confirmed by using a Hewlett Packard 5890 GC–MS. FAME were separated using an Ultra-2 (Hewlett Packard) apolar column (25 m length × 0.22 mm internal diameter × 0.33 μm film thickness). The following temperature program used: 1 min at 60°C, then a linear increase from 60°C to 300°C at 10°C/min, and held at 300°C for 5 min. Helium was used as carrier gas. The mass spectrometer used electron ionization (EI) with filament potential of 70 eV and temperature of 100°C, and a quadrupole mass analyzer in rf/DC mode. Components of the sample were identified by comparison with data from mass spectrometry libraries.

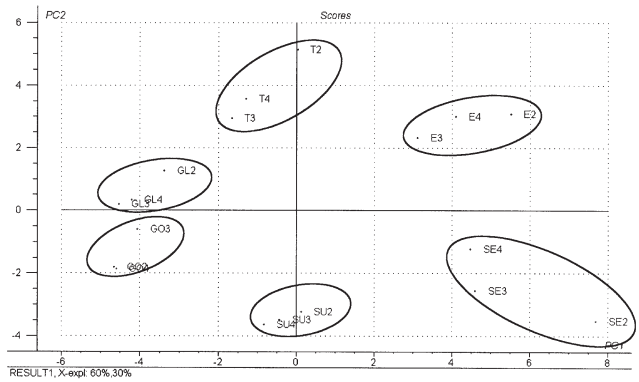

FIG. 1. PCA of the data in Table 1. Data for three samples of each species are shown. T2, T3, and T4: *C. trichotoma* Vell.; E2, E3, and E4: *C. ecalyculata* Vell.; SE2, SE3, and SE4: *C. sellowiana* Cham.; SU2, SU3, and SU4: *C. superba* Cham.; GO2, GO3, and GO4: *C. goeldiana* Huber; GL2, GL3, and GL4: *C. glabrata* DC. Variables analyzed: place of collection, total lipid content (%), FFA content (%), and major lipid classes (% of total).

Statistical analysis. One-way ANOVA and Tukey's studentized range test were used to determine the differences in mean values for each sample based on data collected from three replications of each sample. Significance was established at $P \leq 0.05$.

Principal component analysis (PCA) of the data in Table 1 was performed using the 2.60 version of Pirouette software from Infometrix (Woodinville, WA), in order to group the samples. The data was preprocessed using autoscale and the PCA method was run.

RESULTS AND DISCUSSION

The total lipid contents, FA contents, and contents of the main classes of lipids detected in the mature seeds of six *Cordia* species are shown in Table 1. The total lipid content varied between 1.9% and 13.2%, there being significant differences between the results found in different years for each species and between the species analyzed. Based on these results, the samples can be divided into three groups: those with total lipid content of up to 2.5% total seed weight (*C. glabrata* and *C. goeldiana*); those with total lipid content between 3% and 6% (*C. superba* and *C. trichotoma*), and those with total lipid contents over 7% (*C. ecalyculata* and *C. sellowiana*).

Of these, *C. ecalyculata* and *C. sellowiana* seeds collected in 2002 had significantly higher total lipid content than the seeds collected in the following years (2003 and 2004). For *C. superba, C. glabrata,* and *C. trichotoma,* the same trend was observed, but not for *C. goeldiana.* The differences, however, were not statistically significant in relation to both of the following years. It is known that different climatic conditions, such as rainfall, temperature, soil, and stage of germination of the seeds, can cause significant variations in the lipid contents and composition of seeds of the *Boraginaceae* family (23,24,25). According to meteorological bulletins reported by the governmental agency INPE (National Space Research Institute of the Brazilian Science and Technology Ministry), the year 2002 was the hottest since 1860, and rains were below average in most of Brazil the same year. On the other hand, the years 2003 and 2004 presented higher rainfall than usual for the state of São Paulo (26). Apparently a hotter and drier climate resulted in higher lipid content, although other factors may also be involved in this result.

PCA of the data in Table 1 was run using the following as variables: the place of collection, total lipid content (%), FFA content (%), and the percentages of each lipid class (PL, GL, and NL) for each sample analyzed. The samples were grouped according to their species, indicating that the differences between species were greater than variations between years within each species due to differences in meteorological conditions. PC1 explains 60% of the variability, and PC2 explains 30%.

In relation to total lipid content, the results obtained for *C. superba* (4.4–6.0%) and *C. trichotoma* (3.1–4.3%) seeds are similar to those found by Kleinman *et al.* (16) for seeds of *C. obliqua* Wild (4.0%) and by Mukarram *et al*. (17) for *C. myxa* (3.0%) seeds. Other authors reported higher lipid contents: Daulatabalad *et al.* (15) found 18.0% for *C. rothii.* Mayworm *et al*. (18) reported 19.6% lipid content for seeds of *C. glabrata* A. DC. from the semi-arid region of the Brazilian northeast. A recent study (11) reported 25.8% lipid content in fresh *C. sellowiana* seeds collected in the same region at the same time of the year as Mayworm *et al.* (18). Some plants of the Boraginaceae family are known for their high lipid contents, especially *Myosotis discolor* Pers. (34.1%) and *M. sylvatico* Hoffm. (31.8%) (12). Guil-Guerrero *et al.* (27) analyzed 20 species collected in Spain belonging to the Boraginaceae family and reported lipid contents of 6.6% for *Echium humille* and 30.7% for *Borago officinalis*, but no samples of *Cordia* were analyzed.

The contents of FFA varied between 2.0% and 7.9% of total lipids. NL were the largest class, making up between 89.6–96.4% of the total lipids, the PL were the second largest class with 3.0–8.9% of the total lipids, and the GL the smallest class (0.6– 3.4%). The presence of GLA vas determined in each class of lipids; it is predominant in the NL, making up 95–98% of the FA content of this class of lipids (data not shown). To our knowledge no previous studies have reported the classes of lipids found in seeds of *Cordia.* These results are comparable to those obtained by Senanayake and Shahidi (25) for *Borago officinalis* L. seeds, another member of the same family (Boraginaceae), with an average of 84.1% of NL, 8.6% of PL, and 7.3% of GL in relation to total lipids. The results of Hamrouni *et al.* (24) differ, as they did not report the presence of glycolipids for *Borago officinalis* L seeds.

Table 2 presents the relative composition of the FA found in the six species of *Cordia*. In all species, oleic acid is the predominant FA, ranging from 37% to 48%, followed by linoleic acid (17–25%) and palmitic acid (15–20%). Alpha-linolenic (18:3n-3) and stearidonic (18:4n-3) acids were present in all analyzed *Cordia* species. GLA contents ranged from 1.2% in *C. sellowiana* to 6.8% in *C. ecalyculata*, and was not found in *C. trichotoma*. The variations in GLA content between seeds of the same species collected in different years were not significant at $P = 0.05$.

The predominance of oleic acid was also reported by Mayworm *et al.* (18) for seeds of *C. glabrata* A. DC. (40.8% of total FA), a species from the northeast of Brazil. Other authors have reported very different FA compositions in other species of *Cordia*. In the seed oil of *C. rothii*, linoleic acid was the main FA (40%), followed by palmitic (32.8%), ricinoleic acid (10.8%), and oleic acid (7.8%) (15). Alpha-linolenic acid (24.1%), eicosenoic acid (17.5%), and arachidic acid (8.7%) were found to be predominant in the FA composition of *C. myxa* seeds by Mukarram *et al.* (17), who did not report the presence of GLA in these seeds. Presence of GLA was also not reported in seeds of *C. rothii* (syn. *C.angustifolia)* (15) collected in India, nor in *C. salicifolia* (syn. *C. ecalyculata*) and *C. verbenacea* collected in Beltsville, Maryland (16), nor in seeds of *C.glabrata* A. DC. from the northeast of Brazil (18). Only low concentrations of GLA were found for seeds of *C. obliqua* Wild (0.1%) collected in Beltsville, Maryland, by Kleinman *et al.* (16). However, in a previous study carried out

TABLE 2
FA Composition of the *Cordia* L. Seeds Analyzed

Species	Collection date	FA composition (% of total FA)											
		14:0	16:0	16:1n-7	18:0	18:1n-9	18:2 n-6	18:3n-3	18:3n-6	18:4n-3	SFA	MUFA	PUFA
C. *ecalyculata* Vell.	April 2002	2.1 ± 0.6	18.3 ± 0.2	1.0± 0.6	5.2 ± 0.6	45.1 ± 1.0	17.2 ± 0.9	3.6 ± 0.2	6.2 ± 0.5	1.3 ± 0.3	25.6	46.1	28.3
	April 2003	1.9 ± 0.5	15.0 ± 0.7	1.3± 0.3	4.9 ± 0.5	46.8 ± 0.5	18.2 ± 0.8	4.0± 0.3	5.7± 0.4	2.2 ± 0.5	21.8	48.1	30.1
	April 2004	1.5 ± 0.5	14.6 ± 0.7	1.1 ± 0.2	5.0 ± 0.9	47.6 ± 0.9	19.7 ± 0.9	2.3 ± 0.5	6.8 ± 0.7	1.4 ± 0.2	21.1	48.7	30.2
C. *glabrata* DC.	October 2002	1.8 ± 0.5	14.9 ± 0.5	1.8 ± 0.4	7.5 ± 0.8	42.9 ± 0.6	21.6 ± 1.2	3.0 ± 0.6	3.8 ± 0.4	2.7 ± 0.3	24.2	44.7	31.1
	October 2003	1.2 ± 0.4	19.8 ± 0.5	1.5± 0.3	6.9 ± 1.5	37.1 ± 1.5	24.0 ± 0.9	2.5 ± 0.5	4.8 ± 0.5	2.2± 0.3	27.9	38.6	33.5
	October 2004	1.1 ± 0.5	17.4 ± 0.5	1.0 ± 0.5	6.2 ± 1.3	42.2 ± 0.7	23.4± 0.7	2.3± 0.4	3.9 ± 0.5	2.5 ± 0.4	24.7	43.2	32.1
C. *goeldiana* Huber	December 2002	1.6 ± 0.5	14.6 ± 0.5	1.9 ± 0.4	8.1 ± 0.4	40.4± 0.6	23.0± 1.3	2.3± 0.3	5.2 ± 0.4	2.9 ± 0.5	24.3	42.3	33.4
	November 2003	2.0 ± 0.4	16.9 ± 0.5	1.5 ± 0.4	4.0 ± 0.5	44.8± 1.2	20.3 ± 1.2	3.2 ± 0.5	5.8 ± 0.3	1.5 ± 0.4	22.9	46.3	30.8
	December 2004	2.1 ± 0.6	15.8 ± 0.6	1.7± 0.5	6.3 ± 0.7	41.2± 0.7	22.4 ± 0.3	2.5 ± 0.5	5.5 ± 0.8	2.5 ± 0.5	24.2	42.9	32.9
C. *sellowiana* Cham.	October 2002	1.8 ± 0.5	18.0± 0.8	2.3± 0.2	7.0 ± 0.7	42.3± 1.5	23.7 ± 0.4	2.7± 0.4	1.2 ± 0.4	1.0 ± 0.3	26.8	44.6	28.6
	September 2003	2.1 ± 0.4	18.1± 0.3	1.8 ± 0.4	6.8 ± 0.4	39.8± 0.8	25.3 ± 0.7	3.4± 0.3	1.9 ± 0.4	0.8 ± 0.3	27.0	41.6	31.4
	October 2004	1.3 ± 0.4	17.6± 0.5	1.7 ± 0.7	6.6 ± 0.5	43.9± 0.7	24.3 ± 0.6	2.4± 0.3	1.2 ± 0.3	1.0 ± 0.2	25.5	45.6	28.9
C. *superba* Cham.	September 2002	1.4 ± 0.5	17.3 ± 0.6	2.3 ± 0.5	4.7 ± 0.5	44.3± 0.8	21.8± 0.4	2.9 ± 0.2	2.8 ± 0.4	2.5 ± 0.5	23.4	46.6	30.0
	September 2003	1.3 ± 0.4	16.7 ± 0.5	1.9 ± 0.5	5.6 ± 0.8	46.9 ± 1.3	20.3 ± 1.1	2.2 ± 0.2	3.6 ± 0.5	1.5 ± 0.4	23.6	48.8	27.6
	October 2004	1.4 ± 0.4	17.9 ± 0.8	2.6 ± 0.6	4.8 ± 0.7	45.9 ± 0.8	20.2 ± 0.8	3.1 ± 0.2	2.4 ± 0.5	1.7 ± 0.5	24.1	48.5	27.4
C. *trichotoma* Vell.	June 2002	2.3 ± 0.5	15.1± 0.2	1.8 ± 0.5	8.9 ± 0.9	46.2 ± 0.9	20.4 ± 0.5	2.3 ± 0.4	—	3.0 ± 0.5	26.3	48.0	25.7
	June 2003	1.7 ± 0.6	16.3± 0.5	1.2 ± 0.8	7.1 ± 0.5	46.8 ± 1.5	20.5 ± 0.8	3.3 ± 0.4	—	3.1 ± 0.5	25.1	48.0	26.9
	June 2004	1.4 ± 0.6	18.5± 0.5	1.1 ± 0.5	7.8 ± 0.9	47.0 ± 1.2	17.9 ± 0.7	3.2 ± 0.5	—	3.1 ± 0.4	27.7	48.1	24.2

by our group, GLA was found in seeds of *C. ecalyculata*, *C. myxa,* and *C. sellowiana* collected in Brazil in 2001, ranging from 0.6% to 2.5% of total FA (11). These results suggest that differences in place and time of collection might significantly influence the FA composition of the seeds, although not as much as genetic differences. More detailed studies as to the conditions that result in higher contents of specific FA should be carried out if these seeds are used commercially for the extraction of GLA or other FA.

The concentrations of linoleic, alpha-linolenic, and stearidonic acids and GLA have been indicated as having special taxonomic importance within the Boraginaceae family (12,13,27). Nine species of Boraginaceae from Mongolia (Central Asia) analyzed by Tsevegüren and Aitzetmüller (28) contained GLA (ranging from 6.6% to 13.0%) and stearidonic acid (ranging from 2.4% to 21.4%). *Borago officinalis* L. was found to contain 18.5–24.5% of GLA, but only 0.2– 0.3% of stearidonic acid (12). Another species of Boraginaceae worthy of mention is the plant known in Brazil as confrey (*Symphytum officinale*), as it contains 26–27% of GLA (16,29).

As a previous study indicated, Brazilian *Cordia* species are sources of GLA, a biologically important FA, and five of the six species tested contained GLA in different proportions. The presence of GLA was determined in each class of lipids, and it was found to be predominant in the NL. This is, to our knowledge, the first study on the classes of lipids found in mature seeds of species of *Cordia* found in Brazil.

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Insulin Status-Dependent Alterations in Lipid/Phospholipid Composition of Rat Kidney Microsomes and Mitochondria

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ABSTRACT: Early and late effects of alloxan-diabetes on lipid/phospholipid composition in rat kidney microsomes and mitochondria were examined. In microsomes, early diabetic state resulted in an increase in contents of total phospholipids (TPL), cholesterol (CHL), with an increase in the lysophospholipids (Lyso), phosphatidylcholine (PC), and phosphatidylinositol (PI) components. The sphingomyelin (SPM), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA) content decreased. Treatment with insulin had no effect on PC but PE increased and the other components decreased. In the 1-month diabetic group PI, PS, PE, and PA components decreased, whereas Lyso and PC increased. Treatment with insulin had restorative effects on PE, PI, and PS; Lyso was further elevated whereas PA decreased. In mitochondria, at an early stage of diabetes marginally increased CHL content was restored by insulin treatment. Long-term diabetes lowered the TPL and elevated the CHL content. Treatment with insulin partially restored the TPL and CHL content. A diabetic state decreased the proportion of PE and diphosphatidylglycerol (DPG) components but increased the Lyso, SPM, PC, PI, and PS components in the mitochondria. Treatment with insulin had a partial restorative effect. The membrane fluidity of both microsomes and mitochondria decreased in general in the diabetic condition and was not corrected by insulin treatment at a late stage. However, at an early stage, treatment with insulin fluidized both membranes.

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Diabetic nephropathy is a major cause of diabetes-related morbidity and mortality (1,2). Diabetic nephropathy affects about 15–25% of all type 1 diabetic patients and 20–40% of all patients with type 2 diabetes (3,4).

In our earlier studies we have observed that streptozotocin-diabetes and subsequent treatment with insulin significantly altered the oxidative energy metabolism in kidney mitochondria (5). We have also observed that alloxan-diabetes and subsequent treatment with insulin resulted in significant changes in the kinetic properties of kidney mitochondrial FoF_1-ATPase and succinate oxidase (6,7). Simultaneous studies on kidney microsomes revealed that the kinetic properties of Na^+,K^+-ATPase and of glucose-6-phosphatase were altered in alloxan-diabetes and by subsequent treatment with insulin (Patel, S.P., and Katyare, S.S., unpublished data). The aforementioned mitochondrial and microsomal membrane-bound enzymes are known to have a requirement for specific phospholipids (9–13).

It was therefore of interest to find out the effect of insulin status on the lipid/phospholipid makeup of kidney microsomes and mitochondria. To this end, we have examined the early and late effects of alloxan-diabetes and subsequent treatment with insulin on the lipid/phospholipid profile of rat kidney microsomes and mitochondria. These results are summarized here.

EXPERIMENTAL PROCEDURES

Chemicals. Sodium salt of ethylenediaminetetraacetic acid (EDTA), 1,6 diphenyl-1,3,5-hexatriene (DPH), and bovine serum albumin fraction V (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Silica gel G was purchased from E. Merck (Darmstadt, Germany) and NPH insulin (40 U/mL) was obtained from Eli Lilly (Fegersheim, France). All other chemicals were of analytical-reagent grade and were purchased locally.

Animals. Adult male albino rats of Charles-Foster strain weighing between 200 and 250 g were used. The animals were fasted overnight and the diabetic state was induced by injecting 12 mg of alloxan/100 g of body weight subcutaneously (s.c.) (14–16). Alloxan solutions were prepared freshly in saline. The control rats received an equivalent volume of the vehicle. The early and late effects were examined at the end of 1 week and 1 month of induction of a diabetic state to ascertain the early-onset and long-term effects (5,17). A diabetic state was confirmed in terms of hyperglycemia, polyuria, and glucosuria as detailed previously (5,14–16). For 1-week studies the diabetic animals received insulin from the fifth day of induction of diabetes for three consecutive days, and for 1-month studies diabetic animals received insulin starting from the fourth week of induction of diabetes for seven consecutive days at a dose of 0.8 units of NPH insulin/100 g of body weight twice daily (around 7:00 AM and 6:00 PM) by s.c. route (14–16).

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Abbreviations: CHL, cholesterol; DPG, diphosphatidylglycerol; DPH, 1,6 diphenyl-1,3,5-hexatriene; Lyso, lysophospholipids; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; s.c., subcutaneously; SPM, sphingomyelin; TPL, total phospholipids.

Isolation of mitochondria and microsomes. Isolation of kidney mitochondria was essentially according to the procedures described earlier with some modifications employing the isolation medium comprising 0.25 M sucrose, 10 mM Tris-HCl buffer pH 7.4, and 1 mM EDTA; 250 μg of BSA/mL isolation medium was included (19–21). The postmitochondrial supernatant was subjected to further centrifugation at 12,000 × *g* for 10 min for sedimenting the light mitochondrial fraction. The resulting supernatant was then centrifuged at 100,000 × *g* for 1 h for sedimenting the microsomal fraction. The pellet was washed once by resuspending and resedimenting (22).

The purity of mitochondrial fraction was ascertained by measuring the glucose-6-phosphatase (G6Pase) (a microsomal marker enzyme) activity (Patel, S.P., and Katyare, S.S., unpublished data) and microsomal contamination was found to be less than 2%. The succinate-DCIP-reductase (SDR) activity was not detectable in the microsomal fraction, indicating that there was no mitochondrial contamination. Lactate dehydrogenase (LDH) activity in both microsomal as well as mitochondrial fractions was undetectable, thereby ruling out cytosolic contamination.

Analytical methods. (i) Lipid extraction. Extraction of lipids from the kidney microsomes and mitochondria was carried out essentially according to the method of Folch *et al.* with some modifications as described previously (23,24). Thus briefly, aliquots of microsomal or mitochondrial suspensions containing 4–6 mg of proteins were extracted with 4.0 mL of freshly prepared chloroform/methanol mixture (2:1 vol/vol). The tubes were vortex mixed vigorously, allowed to stand, and after centrifugation at 2,000 rpm for 10 min the organic phase was removed. The samples were then reextracted with 2.0 mL of chloroform/methanol mixture and the organic phase was removed after centrifugation. To the pooled extract 0.2 vol of 0.017% (wt/vol) $MgCl_2$ solution was added and the tubes were vortex mixed. Phase separation was achieved by centrifugation at 3,000 rpm for 10 min and the lower organic phase was carefully removed using a broad gauge needle attached to a syringe, taking care to avoid protein and proteolipid interphase. The organic phase was completely evaporated under a stream of nitrogen and the lipids were redissolved in a known volume of chloroform/methanol mixture (2:1 vol/vol). Suitable aliquots were taken for the determination of total phospholipid (TPL) (25), cholesterol (CHL) (26), and for separation of individual phospholipids by TLC (24,27).

(ii) Separation of phospholipids by TLC. Separation of phospholipid classes was carried out by one-dimensional TLC (24,27). The conditions for preparation of TLC plates, chamber saturation, and so forth were according to the procedure described earlier by Stahl (28). The solvent system used was chloroform/methanol/acetic acid/water (25:15:4:2, by vol) (27). After the run was completed the plates were left at room temperature to remove the solvents. The spots of individual phospholipids were visualized after brief exposure to iodine vapor and marked.

(iii) Estimation of phospholipid phosphorus. After scraping the individual phospholipid spots, the samples were digested with 0.5 mL of 10 N sulfuric acid by heating in a sand bath for 2.5 h. The tubes were allowed to cool and a drop of 70% perchloric acid was added. The tubes were then heated in the sand bath for 25 h to ensure that optically clear samples are obtained and that simultaneously the traces of perchloric acid are removed. The samples taken for estimation of total phospholipid content were also treated in the similar manner. The estimation of phospholipid phosphorus was according to the method described by Bartlett (25).

Membrane fluidity measurements were carried out at 25°C spectrofluorophotometerically using DPH as the probe (24).

Estimation of protein was by the method of Lowry *et al.* using BSA as the standard (29).

Regression analysis was carried out using Jandel Sigmastat Statistical Software, version 2.0.

Statistical evaluation of the data was by Student's *t* test.

RESULTS

Alloxan-diabetes resulted in 15% and 38% reduction in the body weight, respectively, at the end of 1 week and 1 month (Table 1). Treatment with insulin had a marginal restorative effect in the 1-week group while in the 1-month group insulin treatment almost completely restored the body weight (Table 1). At the end of 1 week and 1 month the kidney weight of the

TABLE 1
Effect of Alloxan-Diabetes and Subsequent Treatment with Insulin on Body and Kidney Weight and Serum Glucose

Treatment period	Group	Final body weight (g)	Kidney weight		Serum glucose (mM)
			g	% of body weight	
One week	Control (8)	247.1 ± 5.2	1.81 ± 0.03	0.73 ± 0.021	4.31 ± 0.21
	Diabetic (6)	210.0 ± 6.3^{c}	2.08 ± 0.04^{d}	0.99 ± 0.014^{d}	14.01 ± 0.58^{d}
	Diabetic + insulin (8)	231.3 ± 4.9^{a,§}	1.99 ± 0.06^{c}	0.86 ± 0.022^{c,¶}	6.21 ± 0.32$^{d,\Psi}$
One month	Control (6)	283.7 ± 5.7	1.89 ± 0.04	0.67 ± 0.024	4.61 ± 0.29
	Diabetic (6)	175.8 ± 4.9^{d}	2.55 ± 0.06^{d}	1.45 ± 0.029^{d}	18.87 ± 0.94^{d}
	Diabetic + insulin (6)	279.5 ± 7.2$^{\Psi}$	2.01 ± 0.03^{d,¶}	0.72 ± 0.015^{c,¶}	5.62 ± 0.28^{a,¶}

The results are given as mean ± SEM of the number of independent observations indicated in parentheses.
$^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.002$ and $^{d}P < 0.001$ compared with control values.
$^{\S}P < 0.05$; $^{\P}P < 0.01$ and $^{\Psi}P < 0.001$ compared with corresponding diabetic values.

TABLE 2
Effects of Alloxan-Diabetes and Subsequent Treatment with Insulin on Total Phospholipids (TPL), Cholesterol (CHL), TPL/CHL Ratio, and Fluorescence Polarization in Rat Kidney Microsomes

Groups	Treatment	TPL (μg/mg of protein)	CHL (μg/mg of protein)	TPL/CHL (mol:mol)	Fluorescence polarization *P*
One week	Control (8)	268.8 ± 7.34	171.6 ± 4.44	0.79 ± 0.03	0.231 ± 0.009
	Diabetic (8)	344.1 ± 12.0^{c}	252.4 ± 3.65^{c}	0.68 ± 0.02^{b}	0.280 ± 0.004^{c}
	Diabetic + insulin (8)	352.2 ± 12.3^{c}	324.5 ± 7.16$^{c,\Psi}$	0.54 ± 0.02$^{c,\Psi}$	0.135 ± 0.003$^{c,\Psi}$
One month	Control (6)	272.9 ± 6.98	174.9 ± 3.08	0.78 ± 0.02	0.242 ± 0.007
	Diabetic (8)	285.9 ± 8.51^{b}	279.5 ± 7.39^{c}	0.51 ± 0.02^{c}	0.263 ± 0.004^{a}
	Diabetic + insulin (6)	280.6 ± 8.71^{b,§}	241.5 ± 4.06$^{c,\Psi}$	0.58 ± 0.02^{c,§}	0.283 ± 0.015^{a}

The results are given as mean ± SEM of the number of independent observations indicated in parentheses.
$^{a}P < 0.05$; $^{b}P < 0.02$ and $^{c}P < 0.001$ compared to the corresponding control.
$^{\S}P < 0.05$ and $^{\Psi}P < 0.001$ compared with corresponding diabetic values.

diabetic rats increased by 15% and 35%, respectively. Treatment with insulin had a partial restorative effect only in the 1-month diabetic group. The relative kidney weight increased significantly in the diabetic condition and the increase was more pronounced in the 1-month diabetic animals; insulin treatment had partial restorative effects (data not given). These results are consistent with our previously reported observations (5,14).

Compared to the corresponding controls, the levels of glucose in the serum increased by 3.3- and 4.1-fold in the two diabetic groups. Although insulin treatment lowered the serum glucose levels, the values were always somewhat higher (6.21 and 5.62 mM, respectively) compared to those in the corresponding controls (Table 1). Polyuria in the diabetic state was reflected in terms of 21- and 27-fold increases in the urine volume respectively in two diabetic groups (data not shown). Insulin treatments restored the urine volume to normality (2.2–2.7 mL/24 h). The urinary sugar excretion amounted to about 1.2–1.3 g/24 h in the two diabetic groups; insulin treatment completely abolished the urinary sugar excretion (data not shown). The results agree well with our previously reported observation (5,6,18,30).

The data in Table 2 show that an early diabetic state resulted in increased TPL and CHL contents (28% and 47% increase, respectively) in the microsomal membranes. Insulin treatment was ineffective in restoring the TPL content while CHL content increased further. The TPL content was unaltered in the late diabetic stage, but the CHL content increased by 59%. Insulin treatment had a marginal restorative effect only on CHL content. The TPL/CHL (mol:mol) ratios decreased in the diabetic groups and remained low after insulin treatment. The membrane fluidity decreased in diabetic animals and treatment with insulin significantly fluidized the membrane in the 1-week diabetic animals, whereas the opposite effect was seen in the 1-month diabetic animals (Table 2).

In the mitochondrial membranes the TPL content did not change in the 1-week diabetic animals, whereas the CHL content increased by 16%. Insulin treatment had no effect on TPL content but restored the CHL content. In the 1-month diabetic group the TPL content decreased by 52% and the CHL content increased by 63%. Insulin treatment partially restored the TPL and CHL contents. The TPL/CHL (mol:mol) ratios decreased in the diabetic groups and insulin treatment was effective in normalizing it only in the early stage. The membrane fluidity decreased in diabetic animals and insulin treatment fluidized the membrane in 1-week diabetic animals (Table 3).

The data on phospholipid composition of microsomal membranes are given in Table 3. An early diabetic state resulted in a

TABLE 3
Effects of Alloxan-Diabetes and Subsequent Treatment with Insulin on Total Phospholipids (TPL), Cholesterol (CHL), TPL/CHL Ratio, and Fluorescence Polarization in Rat Kidney Mitochondria

Groups	Treatment	TPL (μg/mg of protein)	CHL (μg/mg of protein)	TPL/CHL (mol:mol)	Fluorescence polarization *P*
One week	Control (8)	262.4 ± 4.64	91.2 ± 2.71	1.45 ± 0.05	0.207 ± 0.001
	Diabetic (8)	255.0 ± 2.86	106.2 ± 2.86^{b}	1.21 ± 0.04^{a}	0.273 ± 0.004^{b}
	Diabetic + insulin (8)	239.0 ± 9.75	82.33 ± 4.19§	1.46 ± 0.03§	0.124 ± 0.004^{b,§}
One month	Control (6)	267.7 ± 2.57	89.7 ± 2.67	1.51 ± 0.05	0.211 ± 0.002
	Diabetic (8)	129.2 ± 7.47^{b}	146.5 ± 5.90^{b}	0.44 ± 0.02^{b}	0.264 ± 0.012^{b}
	Diabetic + insulin (6)	206.7 ± 11.5^{b,§}	114.6 ± 3.69^{b,§}	0.90 ± 0.03^{b,§}	0.239 ± 0.002^{b}§

The results are given as mean ± SEM of the number of independent observations indicated in parentheses.
$^{a}P < 0.01$ and $^{b}P < 0.001$ compared to the corresponding control.
$^{\S}P < 0.001$ compared to the corresponding diabetic.

TABLE 4
Effects of Alloxan Diabetes and Subsequent Treatment with Insulin on Phospholipid Composition in Rat Kidney Microsomes

	Composition (% of total)					
	One week			One month		
Phospholipid class	Control	Diabetic	Diabetic + insulin	Control	Diabetic	Diabetic + insulin
Lyso	3.08 ± 0.14	3.82 ± 0.26^{a}	0.50 ± 0.02$^{e,\Psi}$	3.44 ± 0.18	6.10 ± 0.53^{e}	7.83 ± 0.58$^{a,\Psi}$
SPM	24.50 ± 0.31	21.80 ± 0.31^{e}	19.90 ± 0.58^{e,¶}	25.10 ± 0.50	27.20 ± 0.91	23.92 ± 0.55‡
PC	35.71 ± 0.45	45.52 ± 0.54^{e}	45.46 ± 0.70^{e}	34.55 ± 0.88	37.27 ± 0.74^{a}	34.82 ± 0.44¶
PI	3.93 ± 0.13	4.69 ± 0.19^{c}	2.15 ± 0.12$^{e,\Psi}$	3.29 ± 0.11	2.33 ± 0.21^{d}	3.03 ± 0.15¶
PS	5.00 ± 0.12	4.30 ± 0.13^{d}	3.64 ± 0.18$^{e,\Psi}$	4.91 ± 0.07	2.45 ± 0.14^{e}	3.96 ± 0.13$^{e,\Psi}$
PE	22.40 ± 0.47	16.11 ± 0.60^{e}	26.58 ± 0.40$^{e,\Psi}$	23.01 ± 0.42	20.10 ± 0.92^{b}	23.01 ± 0.43¶
PA	5.37 ± 0.10	3.73 ± 0.20^{e}	1.77 ± 0.15$^{e,\Psi}$	5.43 ± 0.09	4.49 ± 0.37^{a}	3.54 ± 0.20^{e,§}

The results are given as mean ± SEM of the 6–8 independent observations.
Lyso, Lysophospholipid; SPM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid.
$^{a}P < 0.05$; $^{b}P < 0.02$; $^{c}P < 0.01$; $^{d}P < 0.002$ and $^{e}P < 0.001$ compared to the corresponding control.
$^{\S}P < 0.05$; $^{\P}P < 0.02$; $^{\ddagger}P < 0.01$ and $^{\Psi}P < 0.001$ compared to the corresponding diabetic.

significant increase in lysophospholipids (Lyso), phosphatidylcholine (PC), and phosphatidylinositol (PI) components, whereas sphingomyelin (SPM), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA) components decreased. Insulin treatment had no effect on the PC component, which remained elevated. PE increased beyond the control level after insulin treatment, and there was a further lowering of the other phospholipid classes.

The 1-month diabetic group was characterized by the lowering of PI, PS, PE, and PA components, whereas Lyso increased and PC was somewhat elevated. Insulin treatment completely restored PE and PI with partial restoration of PS; Lyso was further elevated while the opposite effect was noted for the PA component (Table 4).

Analysis of the phospholipid profile of mitochondria revealed that the diabetic state had a general effect of increasing Lyso, SPM, PC, PI, and PS and a tendency to decrease PE and diphosphatidylglycerol (DPG) components. Insulin treatment partially corrected the Lyso and PS composition in both the diabetic groups. Insulin treatment could not correct the PI and PE components in the 1-week group and elevated the latter component in the 1-month group (Table 5).

DISCUSSION

The foregoing results emphasized that the early and late effects of the diabetic state on the microsomal membranes differed considerably and the effects of insulin treatment were also differential. As against this, in the mitochondria, a more or less comparable effect was evident at an early as well as late stage of the diabetic state. However, the effects of insulin treatment differed. In any event, the TPL, the CHL contents, the membrane fluidity, or the phospholipid composition did not reach normality after insulin treatment. However, the failure to fully restore membrane lipid composition and membrane fluidity to baseline (or normality) with insulin treatment may reflect the inability to completely model endogenous insulin secretion with exogenous insulin treatment. The results provide evidence that insulin treatment can control the blood sugar levels and induce specific changes in renal subcellular lipid composition that may mediate certain changes in renal cellular activity. Regression analysis revealed that the kidney weight showed progressive positive correlation with microsomal CHL content, with the values of *r* being +0.544 and +0.976, respectively, for the 1-week and 1-month groups; the combined value of *r* for

TABLE 5
Effects of Alloxan Diabetes and Subsequent Treatment with Insulin on Phospholipid Composition in Rat Kidney Mitochondria

	Composition (% of total)					
	One week			One month		
Phospholipid class	Control	Diabetic	Diabetic + insulin	Control	Diabetic	Diabetic + insulin
Lyso	2.19 ± 0.07	5.99 ± 0.29^{d}	1.59 ± 0.13^{c,§}	2.16 ± 0.07	3.63 ± 0.20^{d}	1.70 ± 0.05^{d,§}
SPM	7.58 ± 0.11	10.30 ± 0.29^{d}	7.25 ± 0.18§	7.55 ± 0.11	10.18 ± 0.40^{d}	11.01 ± 0.36^{d}
PC	38.31 ± 0.63	40.33 ± 0.52^{a}	40.52 ± 0.31^{a}	38.70 ± 0.51	40.25 ± 0.59	36.82 ± 0.54^{a,§}
PI	1.08 ± 0.15	2.90 ± 0.15^{d}	1.10 ± 0.05§	1.09 ± 0.10	2.25 ± 0.10^{d}	2.33 ± 0.11^{d}
PS	1.01 ± 0.06	2.66 ± 0.13^{d}	1.27 ± 0.08^{a,§}	1.01 ± 0.05	4.78 ± 0.17^{d}	5.96 ± 0.22^{d,§}
PE	34.73 ± 0.50	25.27 ± 0.62d	35.68 ± 0.29§	34.61 ± 0.46	27.03 ± 0.88^{d}	32.77 ± 0.33^{b,§}
DPG	15.10 ± 0.33	12.70 ± 0.27^{d}	12.51 ± 0.24^{d}	15.01 ± 0.31	11.82 ± 0.41^{d}	9.41 ± 0.52$^{d,\Psi}$

The results are given as mean ± SEM of the 6–8 independent observations.
DPG, diphosphatidylglycerol.
$^{a}P < 0.05$; $^{b}P < 0.02$; $^{c}P < 0.002$; and $^{d}P < 0.001$ compared to the corresponding control.
$^{\Psi}P < 0.02$; and $^{\S}P < 0.001$ compared to the corresponding diabetic.

both the groups was +0.899. The kidney weight also showed progressive negative correlation with mitochondrial TPL content (r = –0.574 and r = –0.943, respectively, for the 1-week and 1-month groups) and a strong positive correlation with mitochondrial CHL content in the 1-month group (r = +0.789). Whether these lipid changes correlate with renal growth and functional renalopathies associated with diabetes *in vivo* remains to be determined. Insulin has been reported to not always successfully correct certain maladies/pathologies associated with diabetes (31–33), and resistance may be reflected in subcellular responses to the hormone as observed in the present study.

A majority of the phospholipid classes except SPM and DPG are synthesized in the microsomes and transferred to other membrane systems. The latter two are synthesized in the plasma membranes and mitochondria, respectively (34–36). Since the diabetic state affected the contents and composition of lipids/phospholipids in the two membrane systems we examined, it was of interest to find out if a correlation existed between the contents of the TPL, CHL, PC, PI, PS, and PE components in the microsomal and mitochondrial membranes. Regression analysis across the control, diabetic, and insulin-treated diabetic animals revealed that in the 1-week group PI and PE components correlated positively (r = +0.632 and r = +0.811, respectively). On the other hand, in the 1-month group, CHL showed a strong positive correlation (r = +0.800); PS and PE showed weak negative and positive correlation, respectively (r = –0.558 and r = +0.577). The results thus imply that the transfer of these lipid components from microsomes to mitochondria is differentially affected by insulin status at an early and late stage of the disease. Decreased plasma phospholipid transfer protein in diabetes has been reported (37–39). Our results suggest that a similar situation may prevail even within the cell (35).

It is interesting to note that the diabetic state significantly lowered the DPG component in the mitochondria and that insulin treatment was ineffective in restoring these values (Table 5). The role of thyroid hormones in DPG synthesis has been demonstrated (40). It has also been shown that the thyroid hormone levels decreased in diabetes [41–46]. Hence the observed effects may be indirectly due to decreased thyroid hormones level (41–46).

It is true that the changes in the phospholipid composition (Tables 4 and 5) appear to be of small magnitude at times. However, if the content of the individual phospholipid class is computed (by multiplying TPL content with % of the individual phospholipid) the differences get amplified. Thus the changes in the content of individual phospholipids will significantly affect the individual phospholipid/protein ratio in the membrane and will also affect the charge distribution and can have bearing on the function of membrane-bound enzymes since membrane-bound enzymes have a requirement of specific phospholipids for their activity (9–13). That this is the case is exemplified by our observations on mitochondrial energy transduction functions, and changes in the kinetic properties of microsomal Na^+,K^+-ATPase and glucose-6-phosphatase and mitochondrial FoF_1-ATPase referred to previously (6,7,47; Patel, S.P., and Katyare, S.P., unpublished data).

Insulin status–dependent alterations in the lipid/phospholipid composition of reticulocytes, red blood cells, liver and heart microsomes, and in the fatty acid desaturase levels have been reported (12,13,48–51). Likewise, thyroid status–dependent changes in the lipid/phospholipid composition of subcelluar membranes have also been reported (52–55). However, as far as we are aware, insulin status–dependent alterations in lipid/phospholipid composition of subcellular components of kidney have not been reported thus far.

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Lymphatic Recovery of Exogenous Oleic Acid in Rats on Long Chain or Specific Structured Triacylglycerol Diets

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ABSTRACT: Specific structured triacylglycerols, MLM (M = medium-chain fatty acid, L = long-chain fatty acid), rapidly deliver energy and long-chain fatty acids to the body and are used for longer periods in human enteral feeding. In the present study rats were fed diets of 10 wt% MLM or LLL (L = oleic acid [18:1n-9], M = caprylic acid [8:0]) for 2 wk. Then lymph was collected 24 h following administration of a single bolus of ^{13}C-labeled MLM or LLL. The total lymphatic recovery of exogenous 18:1n-9 24 h after administration of a single bolus of MLM or LLL was similar in rats on the LLL diet (43% and 45%, respectively). However, the recovery of exogenous 18:1n-9 was higher after a single bolus of MLM compared with a bolus of LLL in rats on the MLM diet (40% and 24%, respectively, $P = 0.009$). The recovery of lymphatic 18:1n-9 of the LLL bolus tended to depend on the diet triacylglycerol structure and composition ($P = 0.07$). This study demonstrated that with a diet containing specific structured triacylglycerol, the lymphatic recovery of 18:1n-9 after a single bolus of fat was dependent on the triacylglycerol structure of the bolus. This indicates that the lymphatic recovery of long-chain fatty acids from a single meal depends on the overall long-chain fatty acid composition of the habitual diet. This could have implications for enteral feeding for longer periods.

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The specific structured triacylglycerol MLM is composed of medium-chain fatty acids (M) in the *sn*-1,3 positions and a long-chain fatty acid (L) in the *sn*-2 position. The medium-chain fatty acids deliver fast energy due to rapid hydrolysis and portal absorption followed by oxidation in the liver (1–5). The long-chain fatty acids are absorbed as *sn*-2 monoacylglycerols in the enterocytes, reacylated, incorporated into chylomicrons, and become available for oxidation or deposition in organs and tissues (6).

When structured triacylglycerols (a mixture of medium- and long-chain fatty acids on the same glycerol backbone) instead of a physical mixture of medium-chain triacylglycerols and long-chain triacylglycerols are incorporated in the parenteral nutrition for patients after surgery, an improved nitrogen balance and faster plasma triacylglycerols and free fatty acid clearance have been observed (7). Similar results were found in several other studies of patients on parenteral feeding with administration of either long-chain triacylglycerols or structured triacylglycerols for a shorter period (5–10 days) and up to 2 months (8–10).

Herzberg *et al.* (11) revealed that the lymphatic transport of fatty acids increased in rats fed a 10 wt% fat diet of fish oil compared with corn oil. Likewise, Jensen *et al.* (12) observed a higher mesenteric lymphatic transport of linoleic acid (18:2n-6) after administration of MLM compared with a randomized oil (the same fatty acid composition as MLM, but no specific structure at the glycerol backbone) in rats fed 20 wt% fish oil compared with rats fed 20 wt% vegetable oil. Furthermore, the fecal excretion of fat was lower in rats fed structured triacylglycerol MLM compared with a randomized oil for 3 wk (13). These studies indicate that alterations in fat absorption could be caused by variation in the dietary fatty acid composition for several days or weeks. However, only limited information is available regarding the influence of a diet containing specific structured triacylglycerol or long-chain triacylglycerols on the absorption of dietary fatty acids.

Several studies have shown a higher lymphatic transport of long-chain fatty acids after administration of specific structured triacylglycerols compared with randomized oils or long-chain triacylglycerols in rats (14–16). However, the recovery of exogenous fatty acids was not determined in these studies, because an unknown amount of endogenous fatty acids contributes to the lymphatic output (17). The lymphatic recovery of the exogenous fatty acids can be determined by isotope labeling. In support of the above-mentioned lymphatic transport studies we recently found a faster lymphatic recovery of ^{13}C-labeled 18:2n-6, when incorporated in MLM (M = caprylic acid [8:0], L = 18:2n-6) compared with long-chain triacylglycerols (18). In contrast, the lymphatic recovery of long-chain fatty acids after continuous infusion of ^{14}C-labeled LLL was significantly higher than after infusion of two ^{14}C-labeled structured triacylglycerols, MLM and MML (L = 18:2n-6, M = 8:0) (19). A study of oleic acid (18:1n-9) from our laboratory supports the results of Tso *et al.* (19). We found a higher lymphatic recovery of ^{13}C-labeled 18:1n-9 after LLL compared with MLM (M = 8:0, L = 18:1n-9) administration in rats (20). The discrepancy between the studies, where isotope-labeled triacylglycerols were used, remains unclear, but could be due to different experimental designs and fatty acid species.

No studies have determined the lymphatic recovery of ^{13}C-labeled long-chain fatty acids of a single bolus of specific structured triacylglycerol or long-chain triacylglycerols in rats fed lipids with different triacylglycerol structures for several weeks.

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Abbreviations: APs, atom percent of a sample; L = long-chain fatty acid; M = medium-chain fatty acid.

Therefore, in the present study rats were fed a diet with 10 wt% LLL (L = 18:1n-9) or MLM (M = 8:0) for 2 wk. Then the rats received a single bolus of ^{13}C-labeled MLM or LLL and the lymphatic recovery of the exogenous fatty acids was determined. The purpose was to evaluate differences in the lymphatic recovery of exogenous fatty acids due to adaptations to the triacylglycerol structure of the dietary fats and to the acute fat intake (the fat bolus), respectively. We expected to find a faster lymphatic uptake of the long-chain fatty acids incorporated in the MLM bolus and that feeding a diet with MLM would further increase the uptake of long-chain fatty acids due to adaptation to the triacylglycerol structure.

EXPERIMENTAL PROCEDURES

Feeding and housing of rats. Male rats (130 g, Wistar; Møllegården Breeding and Research Centre, Lille Skensved, Denmark) were kept at 21 ± 1°C, a humidity of 50 ± 5%, and a light period from 6:00 h until 18:00 h. They had free access to tap water. After acclimatization rats received the experimental diets containing 10% LLL (high oleic sunflower oil; Aarhus Olie A/S, Aarhus, Denmark) or 10% MLM (see following), 40% corn starch, 20% casein, 14% cellulose, 10% sucrose, 5% mineral mixture, 0.5% vitamin mixture, and 0.5% choline chloride (wt%) for 2 wk. The rats were randomized by weight into the two diet groups and their weight development during the 2 wk of feeding was in the same range (LLL diet: 228 ± 6 g and MLM diet: 228 ± 4 g, NS, *t* test). The subgroups of rats, which received the MLM bolus or LLL bolus in the 2 diet groups, had similar weights at the time of surgery.

Production of specific structured triacylglycerols. The specific structured triacylglycerols were produced by enzyme-catalyzed interesterification as described previously (21). The ^{13}C-labeled specific structured triacylglycerols were produced by batch interesterification of 8:0 (Sigma Aldrich, Steinheim, Germany) and ^{13}C-labeled LLL (Cambridge Isotope Laboratories, [Andover, MA, USA). The 2 components were mixed at a substrate ratio of 8:1 (mol:mol), added immobilized *sn*-1,3 specific lipase (10 wt% of the solution, Lipozyme IM; Novozymes A/S, Denmark), and incubated at 50°C for 5 h with constant stirring. After incubation the solution was filtered to remove the lipase. The ^{13}C-labeled MLM was purified by preparative HPLC (22).

Preparation of the fat bolus. At the beginning of the experiment (see following) rats received an isocaloric bolus of either 0.3 mL of MLM or LLL and 0.3 mL of taurocholate solution (20 mM taurocholate and 10 mg/mL choline). The solution was mixed 2 times for 10 s with a Whirly mixer and sonicated 10 s (M.S.E. 150 watt Ultrasonic Disintegrator; M.S.E. Inc., Crawley, England; settings: power = medium and amplitude = 3). The LLL bolus was added as 5 µL of ^{13}C-labeled LLL and the MLM was added as 5 µL of ^{13}C-labeled MLM. There were 4 experimental groups; rats fed the LLL diet or the MLM diet received a bolus of LLL or MLM.

Analysis of oils. LLL and MLM oils were transmethylated by KOH in methanol (23) for determination of the triacylglycerol fatty acid composition. The triacylglycerol structure illustrated by the fatty acid composition of the *sn*-2 position was determined by Grignard degradation (24) followed by the separation on TLC (Silica gel 60; Merck, Darmstadt, Germany), recovery of the *sn*-2 monoacylglycerol, and KOH methylation.

The fatty acid methyl esters were analyzed by a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard GmbH, Walbronn, Germany) on a fused silica capillary column (SP-2380, 60 m, i.d. 0.25 mm, 0.20 µm film; Supelco Inc., Bellefonte, PA). The injector was 260°C and it was used in the split mode with a split ratio of 1:20. The fatty acids were separated by an initial temperature of 70°C, which was kept for 0.5 min, then raised to 160°C at 15°C/min, at 1.5°C/min to 200°C, which was held for 15 min, and finally raised at 30°C/min to 225°C. This temperature was maintained for 10 min. The temperature of the detector (flame ionization) was 300°C. The fatty acid compositions of the triacylglycerols and of the *sn*-2 position are listed in Table 1. The relatively low mol% of 8:0 indicates that the specific structured oil consisted of MLM and MLL/LLM triacylglycerol species, but the oil will be referred to as MLM.

Animal experiment. The experiment was approved by the Danish Committee for Animal Experiments. At the day of surgery, rats were anaesthetized with 0.055 mL/100 g of body weight Zoletil mixture (consisting of zolazepam [125 mg/10.5 mL], tiletamine [125 mg/10.5 mL], Narcoxyl vet [10 mL of 20 mg/mL], Veterinaria AG, Schweiz and Torbugesic [0.5 mL of 10 mg/mL], Fort Dodge Laboratories, Fort Dodge, IA). A clear vinyl tube (o.d. 0.8 mm, i.d. 0.5 mm; Critchley Electrical Products Pty. Ltd, NSW, Australia) was inserted into the mesenteric lymph vessel (25). The tube was subcutaneously led to the abdomen of the rat, through the skin, and fastened with tape (26). The lymph was collected in a 5-mL plastic bottle taped to the trunk of the rat. To prevent the rats from eating the catheter a plastic collar was placed around the neck of the rat. After surgery, rats were administered 0.05 mL of antidote (Antisedan, Farmos, Finland) intramuscularly and 5 mL of saline water (9 g/L NaCl) subcutaneously to prevent dehydration. Rats were placed in individual plastic cages with free access to tap water and a glucose solution (55 g/L glucose; Sigma Aldrich, Steinheim, Germany). Two to 4 h after surgery, rats

TABLE 1
Fatty acid composition (mol%) of the triacylglycerols (TG) and *sn*-2 monoacylglycerols (2-MG) of the experimental oils[a,b]

	MLM		LLL	
Fatty acids	TG	2-mg	TG	2-MG
	mol (%)			
8:0	23.2	1.8	ND	ND
16:0	2.7	1.8	3.7	0.5
18:0	3.4	2.4	3.2	ND
18:1n-9	65.0	87.7	87.1	93.4
18:2n-6	4.8	6.3	5.9	5.4

[a]Data represent the average of 3 determinations ± SEM.
[b]MLM (L = 18:1n-9, M=8.0), LLL (L=18:1n-9).
ND = not detected.

received 0.2 mL of Torbugesic (diluted 1:10 with sterile water).

The next day, a 5-mL bottle with 0.1 mL of EDTA (10 % [wt/vo]) Na_2-EDTA-$2H_2O$; Merck, Darmstedt, Germany) was fastened to the abdomen of the rat and lymph was collected for an hour ("0-sample"). Afterward rats were fed by gavage a bolus of LLL or MLM. Lymph was collected in 1-h fractions for the next 8 h, a night fraction of 15 h, and a 1-h fraction the next morning beginning at 23:00 h. After 24 h of lymph collection, rats were killed by an overdose of sodium pentobarbital. Until analysis lymph samples were kept at –20°C.

Comparisons of this nonrestrained lymph collection model with the conventional restrained model of lymph collection (27) revealed that the data obtained from the 2 models were comparable (data not shown).

Lymph analysis. The lymph samples were extracted by chloroform and methanol (28) after adding an internal triacylglycerol standard (TG-15:0; Sigma Aldrich). The extract was methylated with the KOH-catalyzed method and analyzed by GC-C-IRMS (gas chromatograph-combustion-isotope ratio mass spectrometry; Delta^{PLUS}; Thermo-Finnigan, Bremen, Germany). The samples were injected at 250°C with a split ratio of 1:15 and separated on a fused silica capillary column (same as previously) with the following temperature program: The initial temperature (70°C) was maintained for 3 min and then raised to 150°C at 15°C/min, to 169°C at 1.5°C/min, to 173°C at 0.5°C/min, to 188°C at 3°C/min, and finally raised to 200°C at 20°C/min and maintained for 14 min. The $^{13}C/^{12}C$ ratio of the fatty acid carbon atoms was quantified. The stability of the mass spectrometer was checked every day by a standard on-off test (18 injections of CO_2) and B-scan (control of all mass values, especially water, nitrogen, and argon) and with CO_2 injections in the beginning and end of each run. Correct determinations of sample $^{13}C/^{12}C$ ratios were checked by standard samples with known values. The individual fatty acids were identified by comparing their retention times with retention times of authentic standards (Nu-Chek Prep Inc., Elysian, MN).

Calculations. The atom percent is used for quantitative description of the ^{13}C enrichment of a sample. The atom percent of a sample (AP_S) is calculated from the ratio of $^{13}C/^{12}C$ of the sample (δ_S) and a reference δ of a specific limestone, Pee Dee Belemnite ($\delta = 0.0112372$) (29, 30):

$$AP_s = \frac{100 * 0.0112372 * (0.001 * \delta_s + 1)}{1 + 0.0112372 * (0.001 * \delta_s + 1)} \quad [1]$$

The lymphatic transport of ^{13}C-labeled 18:1n-9 is calculated by multiplying AP_S with the total lymphatic transport of 18:1n-9 in the sample. The recovery of 18:1n-9 is then found by dividing this result with the amount of administered ^{13}C-labeled 18:1n-9 at the beginning of the experiment.

Statistics. Amounts and ratios are stated as average ± SE values. Average lymph flow in the experimental groups was compared with one-way ANOVA. The accumulated lymphatic recovery of ^{13}C-labeled 18:1n-9 after LLL and MLM administration in rats on the LLL or MLM diet was compared with a Student's *t* test. Likewise, the accumulated lymphatic transport of fatty acids was compared with a Student's *t* test. All statistics were performed with SigmaStat (version 2.03; Jandel Corporation, Erkrath, Germany). The level of significance was $P < 0.05$.

RESULTS

Lymph flow. The lymph flow in the 4 experimental groups did not differ [$P = 0.33$, 1W AN (one-way ANOVA)]. It was 0.39 ± 0.05 mL/h and 0.48 ± 0.04 mL/h in rats fed LLL and administered a single bolus of LLL and MLM, respectively. Likewise, the lymph flow was 0.42 ± 0.03 mL/h and 0.51 ± 0.07 mL/h in rats on the MLM diet and administered a single bolus of LLL or MLM, respectively.

AP of the test bolus and the lymph. The AP of the LLL and MLM fat bolus was 1.18 and 1.17, respectively. The baseline AP (just before administration of the fat bolus) was 1.08 ± 0.00 in all groups. In rats fed the LLL diet the AP of the lymph was 1.17 ± 0.02 and 1.16 ± 0.01 three hours after administration of the LLL and MLM fat bolus, respectively. Likewise, in rats fed the MLM diet, the AP was 1.17 ± 0.01 and 1.15 ± 0.00 three hours after administration of the LLL and MLM fat bolus, respectively. Twenty four hours after fat administration the AP was 1.11 ± 0.01 in all groups.

Recovery of ^{13}C-enriched lymphatic 18:1n-9. The maximum rate of lymphatic recovery of ^{13}C-labeled 18:1n-9 was obtained 2–3 h after administration of the lipid bolus. A similar accumulated lymphatic recovery of ^{13}C-labeled 18:1n-9 was observed in rats on the LLL diet 24 h after administration of an LLL or an MLM lipid bolus (Fig. 1A). In contrast, a higher accumulated lymphatic recovery of exogenous 18:1n-9 was found after 24 h in rats on an MLM diet and administered a bolus of MLM compared with a bolus of LLL (Fig. 1B; $P = 0.009$, *t* test).

There was no difference between the accumulated recovery of ^{13}C-labeled 18:1n-9 after consumption of the MLM bolus in rats on the LLL compared with the MLM diet ($P = 0.64$, *t* test). However, a tendency to reduced recovery of lymphatic ^{13}C-labeled 18:1n-9 was observed in rats fed the MLM diet compared with the LLL diet after administration of an LLL lipid bolus ($P = 0.07$, *t* test).

The ^{13}C-labeled 18:1n-9 was the only enriched fatty acid detected in the lymph samples indicating neither elongation nor chain shortening of the administered fatty acid.

Lymphatic transport of medium-chain fatty acids. 8:0 was detected during the first 8 h of lymph collection in rats, which had received the MLM bolus (Fig. 2). The lymphatic transport of 8:0 peaked 2 h after oil administration in both diet groups with a maximum transport of approximately 1.5 mg/h and no significant differences between groups ($P = 0.10$, *t* test). The accumulated transport of 8:0 eight hours after MLM administration constituted approximately 3% of the administered dose (2.6% in rats on the MLM diet and 4.3% in the rats on the LLL diet).

Lymphatic transport of fatty acids. There were no differences in the accumulated lymphatic transport of both exogenous and endogenous 18:1n-9 in rats fed the LLL diet 24 h after

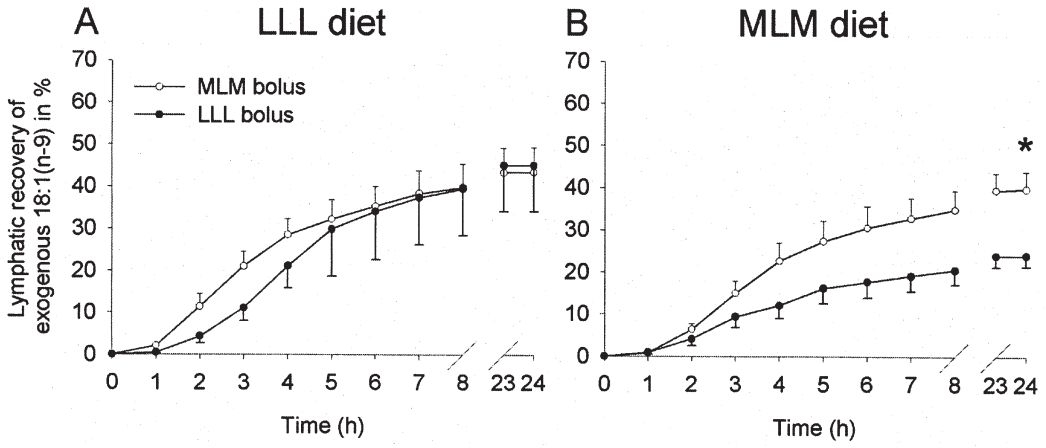

FIG. 1. Recovery of lymphatic 18:1n-9 after feeding bolus of LLL (filled circles) or MLM (empty circles) in rats on (A) LLL diet, $n = 6–9$, and (B) MLM diet, $n = 7–8$. Average ± SEM.

administration of an LLL or an MLM bolus (Fig. 3A; $P = 0.94$, *t* test). Likewise, no differences were observed in the accumulated lymphatic transport of palmitic acid (16:0), stearic acid (18:0), and 18:2n-6 after administration of an LLL or an MLM bolus in rats fed the LLL diet (Fig. 3B–D; $P = 0.15–0.94$, *t* test). However, the lymphatic transport of the endogenous fatty acid 20:4n-6 was higher 24 h after administration of MLM compared with LLL in the rats fed LLL (Fig. 3E; $P = 0.03$, *t* test).

The accumulated lymphatic transport of 18:1n-9 was similar 24 h after LLL and MLM administration in rats fed an MLM diet (Fig. 4A; $P = 0.48$, *t* test). Furthermore, the accumulated lymphatic transport of 16:0 and 18:0 was similar following administration of an LLL or an MLM bolus in rats fed the MLM diet (Fig. 4B–C; $P = 0.32$ and $P = 0.51$, respectively, *t* test). However, the lymphatic transport of 18:2n-6 was higher 24 h after administration of MLM compared with LLL in the rats on the MLM diet (Fig. 4D; $P = 0.03$, *t* test), whereas there was a tendency to a higher accumulated lymphatic transport of the endogenous fatty acid 20:4n-6 after intake of the MLM compared with the LLL bolus (Fig. 4E; $P = 0.06$, *t* test).

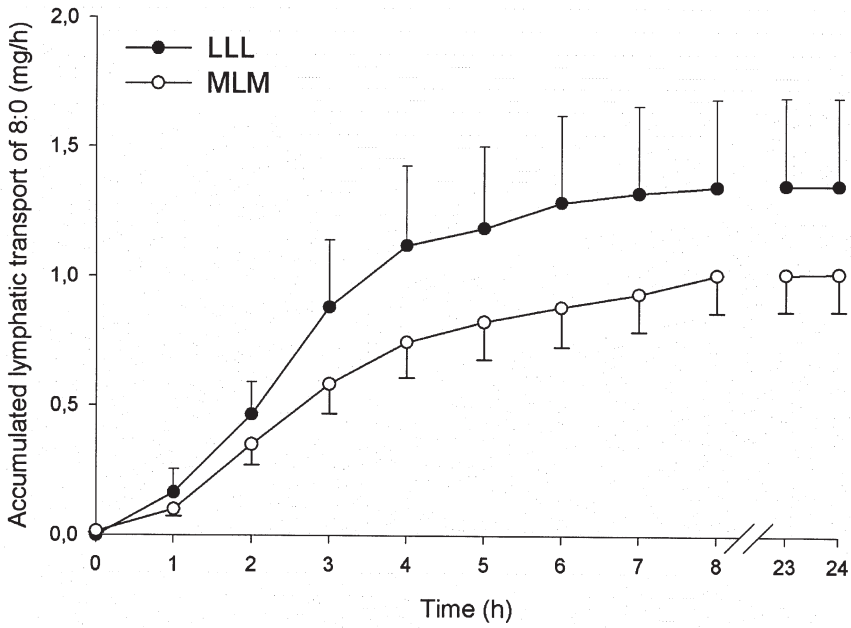

FIG. 2. Lymphatic transport of 8:0 (mg/h) after administration of a single bolus of MLM in rats on the LLL diet (filled circles), $n = 9$, and rats fed MLM (empty circles), $n = 8$. Average ± SEM.

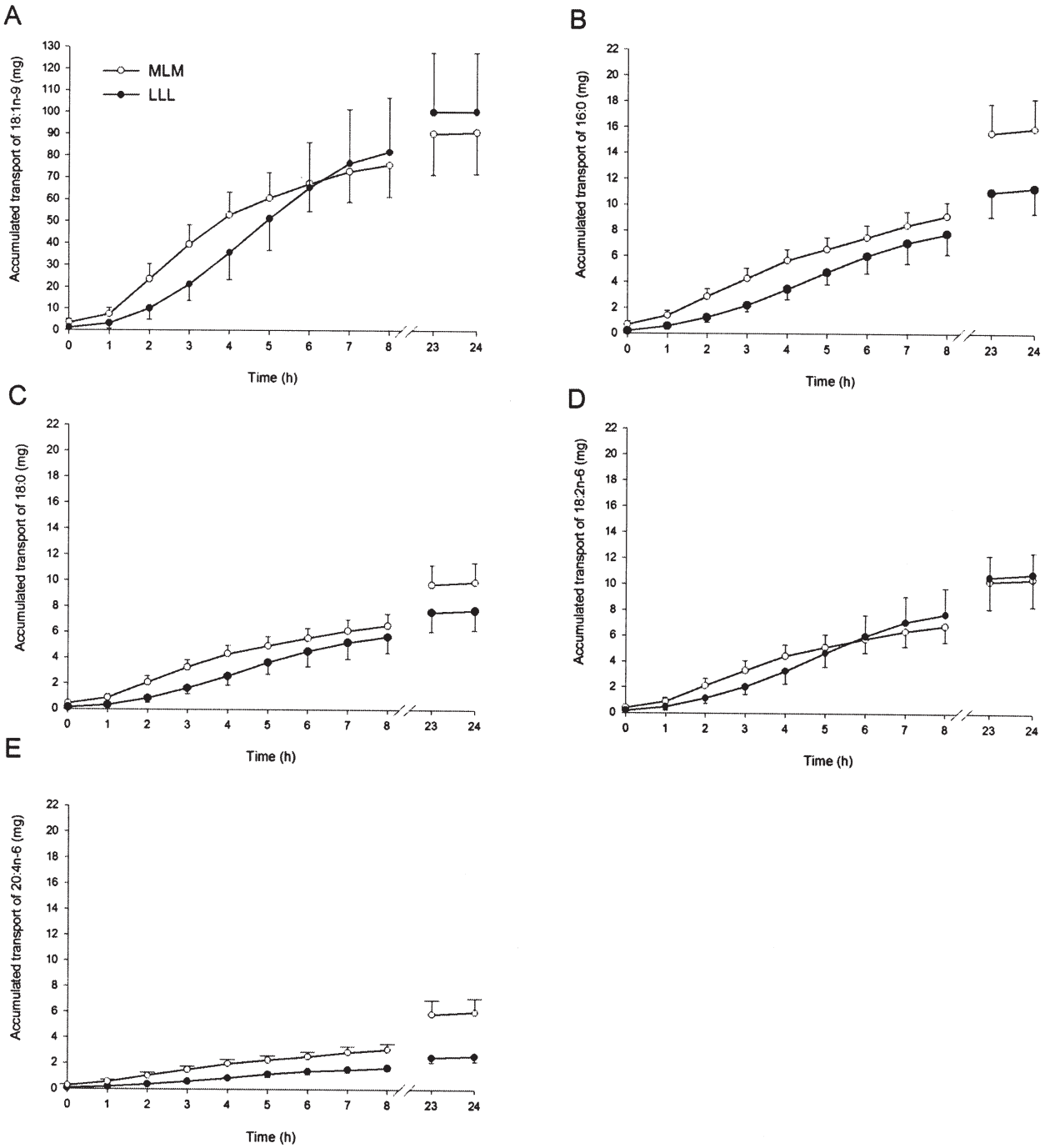

FIG. 3. Accumulated lymphatic transport (mg) of 18:1n-9 (A), 16:0 (B), 18:0 (C), 18:2n-6 (D), and 20:4n-6 (E) following a single bolus of LLL (filled circles) or MLM (empty circles) in rats on the LLL diet, n = 6–9. Average ± SEM.

DISCUSSION

The main finding of the study is that adaptation to a diet containing specific structured triacylglycerol (MLM, M = 8:0, L = 18:1n-9) for 14 days resulted in a higher lymphatic recovery of ^{13}C-labeled 18:1n-9 after administration of a single bolus of MLM compared with LLL. In contrast, rats fed a diet containing LLL had similar lymphatic recovery of exogenous 18:1n-9 after administration of a lipid bolus of MLM or LLL.

The lymphatic recovery of ^{13}C-labeled 18:1n-9 in rats fed the MLM or the LLL diet after administration of a single meal of MLM was at the same level, whereas administration of an LLL bolus in the 2 diet groups tended to reveal a higher lymphatic recovery of ^{13}C-labeled 18:1n-9 in the LLL diet group.

Structured triacylglycerols are used as the lipid component for parenteral feeding and have advantages compared with

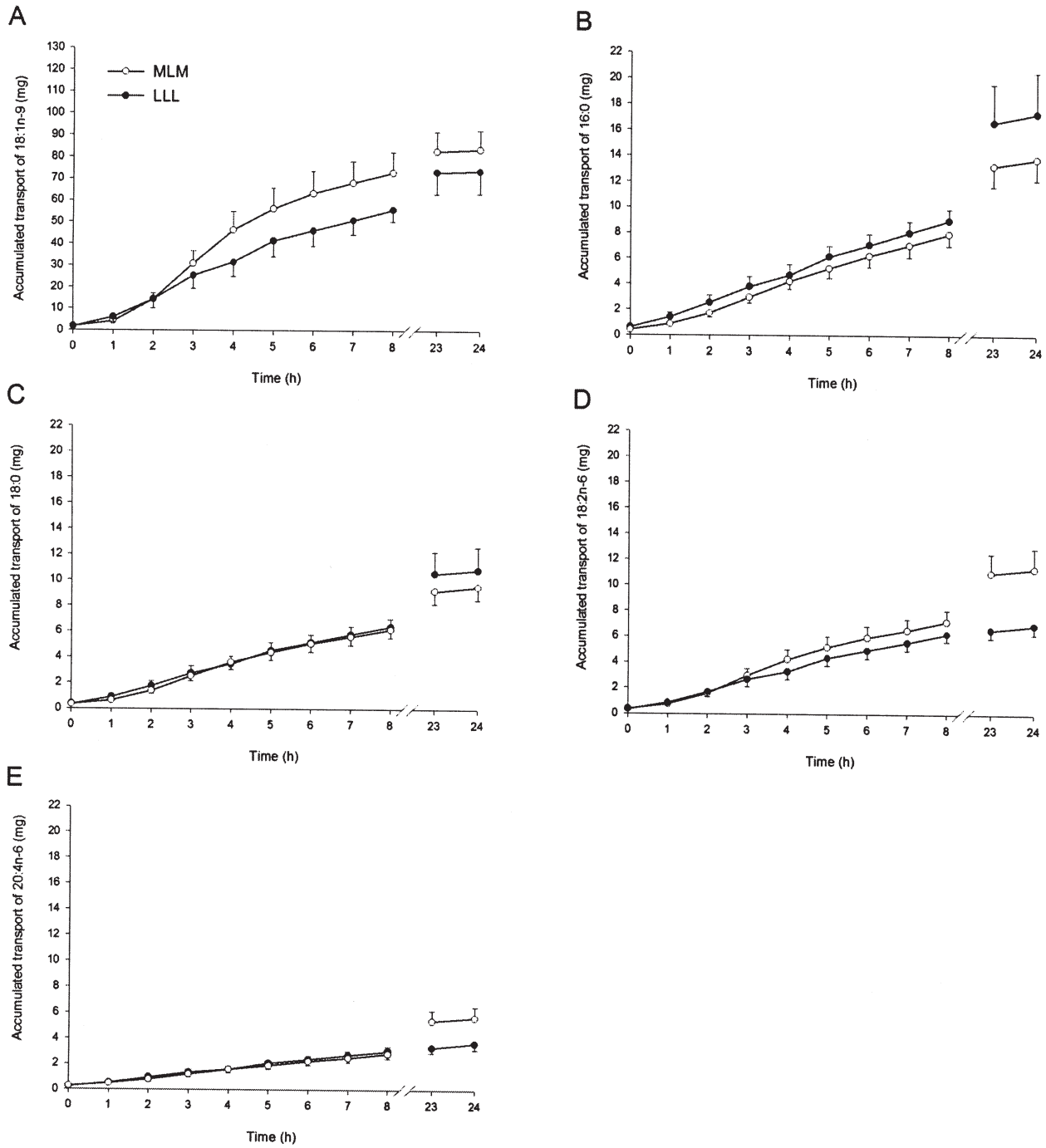

FIG. 4. Accumulated lymphatic transport (mg) of 18:1n-9 (A), 16:0 (B), 18:0 (C), 18:2n-6 (D), and 20:4n-6 (E) following a single bolus of LLL (filled circles) or MLM (empty circles) in rats on the MLM diet, $n = 7–8$. Average ± SEM.

long-chain triacylglycerols (8–10). However, long-term effects on the lipid absorption of a diet containing structured triacylglycerols have not been examined. Our results show that the MLM bolus provided a better absorption of 18:1n-9 compared with the LLL bolus in rats on the MLM diet. Studies without adaptation to specific structured triacylglycerols for a longer period have demonstrated higher (14–16,18) or lower (19,20) lymphatic transport of long-chain fatty acids after administration of MLM compared with LLL or randomized oils.

When MLM is ingested, hydrolyzed, and absorbed, there is a limited amount of exogenous fatty acid available for resynthesis of triacylglycerols in the enterocyte due to the low content of long-chain fatty acids and transport of the medium-chain fatty acids *via* the portal vein. Therefore, endogenous

long-chain fatty acids are mobilized from the intestine and bile and used for reacylation (17). In the present study, rats were allowed to adapt to the specific structured triacylglycerols with low exogenous amounts of long-chain fatty acids for 2 wk before lymph cannulation, which could have induced a higher level of endogenous long-chain fatty acids available for resynthesis of triacylglycerols in the enterocyte and thereby increase the lymphatic recovery of the ingested long-chain fatty acids of the MLM.

Rats, which received the LLL diet during 2 wk exhibited similar lymphatic recovery of ^{13}C-labeled 18:1n-9 after administration of the LLL and the MLM lipid bolus. Nevertheless, the recovery of ^{13}C-labeled 18:1n-9 during the first hours after administration of the lipid bolus (Fig. 1A) revealed a tendency to a faster recovery of 18:1n-9 after administration of the MLM bolus compared with the LLL bolus. This is in line with earlier results from our laboratory, where the lymphatic transport of docosahexaenoic acid (22:6n-3) and eicosapentaenoic acid (20:5n-3) was higher during the first hours after administration of an MLM bolus compared with randomized oil, but after 24 h, the accumulated lymphatic transport was similar without differences in regard to triacylglycerol structure (15). Likewise, a faster lymphatic recovery of ^{13}C-labeled 18:2n-6 was observed in rats after consumption of MLM compared with LLL, but the accumulated recovery after 24 h was the same in both groups (18).

The effect of the 2 diets on the lymphatic absorption of long-chain fatty acids after the single bolus of MLM did not differ. This indicates that the capacity for absorption of this specific structured triacylglycerol was not dependent on the type of diet before ingestion of the MLM bolus. Interestingly, it was observed in both diets that the accumulated lymphatic transport of the endogenous fatty acid 20:4n-6 was higher 24 h after feeding the MLM bolus compared with the LLL bolus (Figs. 3E and 4E) indicating a higher transport of endogenous fatty acids.

The absorption of the LLL bolus after 14 days on the MLM diet tended ($P = 0.07$) to be lower than the recovery of 18:1n-9 in the rats fed the LLL diet. In earlier studies it has been observed that the fat composition of the diet influences the uptake of the long-chain fatty acids of a single meal (11,12). However, in these studies the fatty acid species in the different diets varied considerably, whereas the long-chain fatty acid content of both diets in the present study was 18:1n-9. One could speculate that the lower recovery of 18:1n-9 after administration of the LLL bolus could be due to a higher availability of endogenous fatty acids in rats on the MLM diet as mentioned previously, which would then result in competition between the endogenous and exogenous long-chain fatty acids during absorption. Furthermore, the lower recovery could also be due to MLM diet-induced changes in the intestinal brush border membrane lipid composition or reduced enzyme activity in the hydrolysis and or resynthesis of triacylglycerols in the enterocyte.

The lymphatic recovery of exogenous 18:1n-9 did not exceed 40–45%. This result agreed with the transport of an entirely exogenous fatty acid in the study of Straarup and Høy (31) (20:5n-3: 41%). Likewise, in a study with constant infusion of ^{14}C-labeled LLL and MLM, the lymphatic recoveries of long-chain fatty acid were 62% and 48%, respectively (19). Furthermore, during the first hours of absorption of the fat bolus, the lymph mainly consisted of exogenous fatty acids as indicated by the high AP level of the lymph collected 3 h after fat administration (~1.15–1.17) compared with the AP level of the fat bolus itself (~1.17–1.18).

No other ^{13}C-labeled fatty acids were observed in the lymph, which demonstrated that 18:1n-9 was not elongated and/or desaturated in the enterocyte. The expected fate of the nonessential 18:1n-9 is mainly oxidation in organs and peripheral tissue, which was demonstrated by Watkins *et al.* (32), who observed a higher oxidation of ^{13}C-labeled trioleylglycerol (11%) compared with ^{13}C-labeled 16:0 as free fatty acid (7%) after simultaneous administration of the lipids.

The lymphatic transport of 8:0 was similar in both feeding groups. This revealed that the lymphatic distribution of 8:0 after digestion and absorption of MLM was independent of the diet type. Up to 4.3% of the administered 8:0 was found in the lymph, which is comparable to the findings of Ikeda *et al.* (16) and Mu and Høy (22). The majority of the ingested 8:0 was not esterified in the enterocyte, but transported directly through the cell as free fatty acids and via the portal vein to the liver (3,33).

The MLM oil consisted of different triacylglycerol species (MLM, MLL, and LLM) due to technical limitations during preparation. This implies that a faster and eventually higher recovery of the 18:1n-9 could be expected if it had been possible to produce an oil of pure MLM.

The present study indicates that the dietary fat structure and composition influence fat absorption not only after a single bolus of fat, but also as an adaptation following 14 days with diets composed of long-chain triacylglycerols or specific structured triacylglycerol. We found that a diet containing specific structured triacylglycerol (MLM) enhances the lymphatic recovery of exogenous 18:1n-9 after administration of a single bolus of MLM compared with a bolus of LLL. The implication of this finding for enteral feeding is that specific structured triacylglycerols can be used for longer periods without decreasing the absorption of the long-chain fatty acids of the diet.

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Deoxycholic Acid Formation in Gnotobiotic Mice Associated with Human Intestinal Bacteria

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ABSTRACT: In humans and animals, intestinal flora is indispensable for bile acid transformation. The goal of our study was to establish gnotobiotic mice with intestinal bacteria of human origin in order to examine the role of intestinal bacteria in the transformation of bile acids *in vivo* using the technique of gnotobiology. Eight strains of bile acid–deconjugating bacteria were isolated from ex–germ-free mice inoculated with a human fecal dilution of 10^{-6}, and five strains of 7α-dehydroxylating bacteria were isolated from the intestine of limited human flora mice inoculated only with clostridia. The results of biochemical tests and 16S rDNA sequence analysis showed that seven out of eight bile acid–deconjugating strains belong to a bacteroides cluster (*Bacteroides vulgatus, B. distasonis,* and *B. uniformis*), and one strain had high similarity with *Bilophila wadsworthia*. All five strains that converted cholic acid to deoxycholic acid had greatest similarity with *Clostridium hylemonae*. A combination of 10 isolated strains converted taurocholic acid into deoxycholic acid both *in vitro* and in the mouse intestine. These results indicate that the predominant bacteria, mainly *Bacteroides*, in human feces comprise one of the main bacterial groups for the deconjugation of bile acids, and clostridia may play an important role in 7α-dehydroxylation of free-form primary bile acids in the intestine although these strains are not predominant. The gnotobiotic mouse with bacteria of human origin could be a useful model in studies of bile acid metabolism by human intestinal bacteria *in vivo*.

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Bile acids are transformed by the intestinal microflora. Bile acids in humans are synthesized from cholesterol in the liver, and conjugated to either glycine or taurine. Bile salts are secreted into the small intestine as a component of bile and play an important role in the absorption of lipids. More than 90% of bile acids are reabsorbed, mostly in the ileum and recycled via the enterohepatic circulation. The rest enter the large intestine, where all bile acids (primary and secondary) are deconjugated and primary bile acids such as cholic acid (CA) and chenodeoxycholic acid (CDCA) are further 7α-dehydroxylated to deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. Levels of secondary bile acids including DCA and LCA appear to show a strong correlation with colorectal cancer *in vitro* (1,2) and in animal models (3–5). Epidemiological studies reported that the level of fecal 7α-dehydroxylase activity was significantly higher in colorectal cancer patients than in controls (6–8).

Bile salt hydrolase activity is found in many kinds of intestinal bacteria (9–12). 7α-Dehydroxylating activity has also been reported in certain groups of intestinal bacteria (13). However, in some species, results are contradictory. Some reports indicate that bacterial strains that belong to *Bifidobacterium* (14), *Bacteroides* (14), and *Lactobacillus* (15,16) showed 7α-dehydroxylating activity. By contrast, other groups showed the negative results for the activity in *Bifidobacterium* strains (17,18), *Bacteroides* strains (19,20), or *Lactobacillus* strains (18,21). Only certain strains belonging to *Eubacterium* and *Clostridium* have been examined specifically for their 7α-dehydroxylating activity (22–26). It is considered that the difficulty in isolating 7α-dehydroxylating bacteria from the intestine arises from the small populations of these bacteria in the intestine (27,28), although most of the primary bile acids in the intestine are deconjugated and further transformed into secondary bile acids. The precise mechanism by which the small numbers of bacteria responsible for 7α-dehydroxylation transform most of the bile acids in the intestine remains to be elucidated.

In our previous study, we inoculated human intestinal bacteria with the ability to transform bile acids *in vitro* into germ-free (GF) mice. However, only small amounts of secondary bile acids were observed in the cecal content, indicating that the ability of bacteria to transform bile acids *in vitro* does not necessarily reflect that *in vivo* (29,30). Then we produced various groups of limited-flora mice inoculated with intestinal bacteria of human origin. Approximately 90% of the cecal bile acids were deonjugated when GF mice were inoculated with human fecal dilutions of 10^{-2} or 10^{-6}, or anaerobic growth from a dilution of 10^{-6}. In those ex-GF mice, bacteroidaceae colonized in the intestine at the highest levels, followed by eubacteria and clostridia. In contrast, in the cecal contents of ex-GF mice inoculated only with clostridia, unconjugated bile acids accounted for less than 40% of all bile acids. However, the percentage of DCA in these mice was the same as that in the other groups. These results indicate that the dominant anaerobic bacterial combination is efficient for the deconjugation of primary bile acids, and that clostridia in human feces may play an important

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Abbreviations: EG, Eggerth-Gagnon; EGF, Eggerth-Gagnon Fildes; GB, gnotobiotic; GF, germ-free; MPYG, modified peptone-yeast-glucose; TS, tripticase soy.

role in the 7α-dehydroxylation of unconjugated primary bile acids in the intestine (31). However, the attempt to establish gnotobiotic (GB) mice whose intestinal bile acids consisted of a certain amount of secondary bile acid with predominant bacteria isolated from limited human flora mice was not successful, indicating that bile acid–transforming bacteria are not necessarily the predominant population in the intestine.

The objective of this study was to obtain combinations of human intestinal bacteria that are able to transform conjugated primary bile acids to secondary bile acids *in vivo*, and to produce GB mice with human intestinal bacteria as an *in vivo* model for the analysis of bile acid metabolism by intestinal bacteria.

MATERIALS AND METHODS

Animals. Female GF BALB/c mice (12–15 wk old) were bred in our laboratory under GF conditions in flexible vinyl isolators. Each group of female GF mice was transferred to an autoclavable stainless steel isolator (32) and given a pelleted commercial diet (CMF, Oriental Yeast Co., Tokyo, Japan) sterilized with γ-irradiation at 50 kGy and autoclaved water *ad libitum*. The study was approved by the Laboratory Animal Use and Care Committee of the Faculty of Agriculture, University of Tokyo.

Bacterial strains. Clostridium hiranonis TO-931^T (JCM 10541^T) and *Eubacterium lentum*–like c-25 were provided by Dr. F. Takamine (Laboratory of Microbiology, School of Health Science, Faculty of Medicine, University of Ryukyus, Okinawa, Japan), and by Dr. H. Oda (Department of Bacteriology, Faculty of Medicine, Kagoshima University, Kagoshima, Japan), respectively.

Production of ex-GF mice associated with limited human fecal flora. Ex-GF mice with the ability to convert bile acids in the intestine were produced previously (31). Briefly, 1 g of fresh feces from a healthy human male was immediately placed in 9 mL of an anaerobic diluent (33) under oxygen-free CO_2 gas. A portion was transferred into an anaerobic chamber and serially diluted with anaerobic trypticase soy (TS) broth supplemented with 0.084% Na_2CO_3, 0.05% agar, and 0.05% cysteine hydrochloride monohydrate. Next, 10^{-6} diluted fecal suspensions were prepared for inoculation into GF mice to produce ex-GF mice. Another portion of the 10^{-2} dilution of human feces was treated with chloroform according to the method of Itoh and Mitsuoka (34). A fecal suspension was added to a final concentration of 3% chloroform, was shaken vigorously for about 30 s, and was subsequently incubated with shaking for 1 h at 37°C. After the incubation, chloroform was eliminated from the suspension by percolating with O_2-free N_2 gas. Only bacterial spores survived after this treatment (34).

Each fecal dilution and bacterial suspension (0.5 mL) was orally inoculated into the stomach of GF mice with a stainless steel catheter.

Bile acids. CA, CDCA, ursodeoxycholic acid (UDCA), DCA, LCA, and glycine and taurine conjugates of each of these were obtained from Calbiochem (La Jolla, CA). The following bile acids were obtained from Steraloids (Wilton, NH): hyocholic acid (HCA), hyodeoxycholic acid (HDCA), α-muricholic acid (α-MCA), β-muricholic acid (β-MCA), ω-muricholic acid (ω-MCA), and 3α-12-Oxo (3α12=O). 7-Oxo-deoxycholic acid (7=O DCA) was purchased from GL Science, Inc. (Tokyo, Japan). The following glycine (G)- and taurine (T)-conjugated bile acids were synthesized according to the method of Tserng and Klein (35): G-HCA, T- HCA, G-HDCA, T-HDCA, G-α-MCA, T-α-MCA, G-β-MCA, T-β-MCA, G-ω-MCA, and T-ω-MCA.

In vitro screening of bile acid transforming activity. A modified peptone-yeast extract broth (MPYG) (18,27) was used for culture with some modifications. This medium (100 mL) contained 200 μM CA or TCA, 0.1 g of glucose, 1 g of proteose peptone no. 3, 1 g of trypticase peptone, 1 g of yeast extract, 0.05 g of cysteine hydrochloride, 4 mL of salt solution, 0.02 mL of vitamin K solution, and 1.0 mL of hemin solution dissolved in 0.1 M sodium phosphate buffer (pH 7.5). The medium was dispensed in 2.0-mL aliquots, and transferred to an anaerobic chamber after autoclaving. Bacterial strains isolated from ex-GF mice were pre-incubated in EGF broth (33), and 0.05 mL of each overnight culture was inoculated into the MPYG and incubated for 3–7 d at 37°C in an anaerobic chamber. For the isolation of 7α-dehydroxylating bacteria, a bacterial mixture from agar plate was directly inoculated into MPYG (Fig. 1). After centrifugation at 3,000 rpm for 10 min, 0.5 mL of the supernatant was extracted with 10 mL of absolute ethanol at 90°C for 60 min. After filtration and drying under reduced pressure, the pellet was dissolved in 2.0 mL of methanol/0.1 M dibasic ammonium phosphate (50/50) solution. After filtration through a 0.45-μm filter (Gelman Science, Tokyo, Japan), 10 μL of the solution was used for HPLC analysis.

Cecal bile acid determination. Three weeks after GF mice were orally inoculated with human fecal suspension or mixture of human fecal bacteria, the cecal contents of each mouse were collected, freeze- dried, and pulverized. A 50-mg portion of the dry cecal material was treated three times with 10 mL of absolute ethanol at 90°C for 1 h, and filtered. The extracts were mixed, dried, and suspended in 90% ethanol, subjected to piperidinohydroxypropyl Sephadex-LH-20 column chromatography (Shimadzu Co., Kyoto, Japan) to obtain free, glycine-conjugated, and taurine-conjugated fractions (36).

HPLC conditions. Each of the bile acids was separated using the LC module-1 HPLC system (Waters, MA) with a Symmetry C18 separation column (4.6 mm i.d. × 150 mm, Waters). Bile acids were then detected by the post-column fluorescent method with a 3α-HSD immobilized column (E-3α-HSD, 4.0 mm i.d. × 20 mm, Sekisui Chemical Co. Ltd., Osaka, Japan), β-NAD solution delivered by an A-60-S pump (Eldex Laboratories Inc., CA) and a 474-nm scanning fluorescence detector (Waters) at an excitation wavelength of 340 nm and an emission wavelength of 460 nm (36,37). Each bile acid was identified by its relative retention time compared with that of standard bile acids, and peak heights were calculated automatically on a Waters 805 data station.

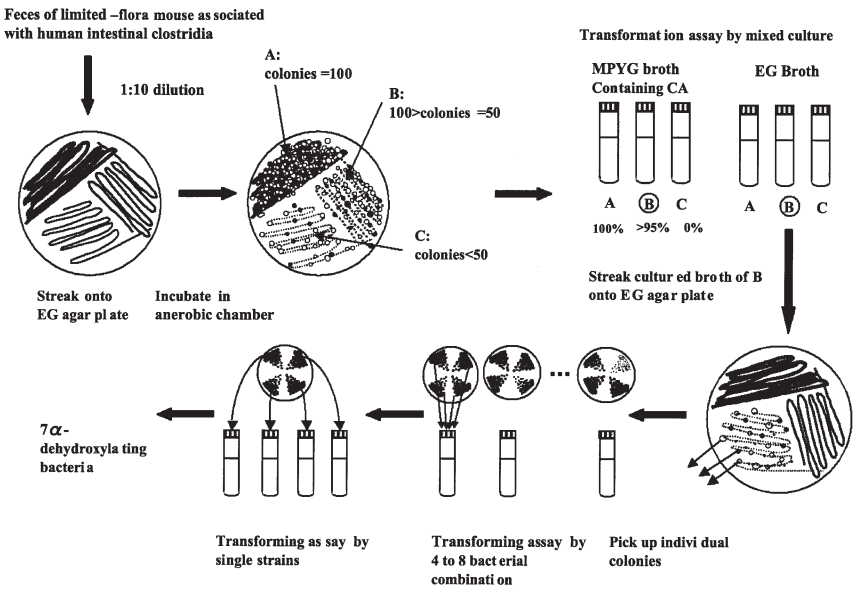

FIG. 1. Isolation of cholic acid 7α-dehydroxylating bacteria from fecal samples of ex-germ-free mice associated with human intestinal clostridia.

Enumeration of fecal bacteria in GB mice. Three weeks following gastric inoculation of a mixed human fecal flora, feces of mice were collected, immediately weighed, and transferred to an anaerobic chamber. They were homogenized with a 50-fold volume (vol/wt) of anaerobic TS broth, serially diluted, and inoculated on EG agar plates. Bacteria were counted and identified by Gram staining, cell morphology, and colony morphology. The numbers of bacteria were expressed as log 10 counts of viable bacteria per gram wet weight of feces.

Isolation of bile acid–transforming bacteria from limited human flora–associated mice. The intestinal microflora of ex-GF mice inoculated with a human fecal dilution of 10^{-6} showed a good ability to deconjugate taurine-conjugated bile acids (31). The feces from these ex-GF mice were diluted in an anaerobic diluent (33), inoculated onto NBGT selective agar plates [bacteroidaceae selective medium; (33)], and incubated for 48 h in an anaerobic steel wool jar filled with 100% CO_2. Twenty-four strains of bacteria grown on NBGT agar plates were isolated and screened for the ability to deconjugate TCA to CA *in vitro.*

Clostridial strains capable of bile acid 7α-dehydroxylation were isolated from ex-GF mice inoculated with human intestinal clostridia, according to the method of Takamine and Imamura (28) with minor modifications. Feces of ex-GF mice were immediately transferred into an anaerobic chamber, and one loopful of a 10-fold dilution in reduced TS broth was directly streaked onto EG agar plates and incubated at 37ºC for 3 d. The developed colonies were grouped in three areas, A (more than 100 colonies), B (between 50 and 100 colonies), and C (less than 50 colonies). The mixed organisms grown in each area of the EG agar plates were inoculated into MPYG broth containing CA for the screening of 7α-dehydroxylation *in vitro*. The remaining organisms were incubated in EG broth at 37ºC for 2 d in the anaerobic chamber and further kept at room temperature. One loopful of the cultured EG broth from an appropriate area was again inoculated onto an EG agar plate and incubated at 37ºC for 2 d in the anaerobic chamber. After incubation, each of the single colonies was isolated and reinoculated onto EG agar plates to obtain single cultures. Four to eight of the single strains were mixed and checked for bile acid 7α-dehydroxylation activity. From the mixed cultures that were positive for 7α-dehydroxylation each of the single strains was screened for 7α-dehydroxylating activity.

Identification and characterization of bacteria. Morphological and biochemical characteristics were determined as described by Kaneuchi *et al.* (38) and Holdmann and Moore (39). The growth in peptone-yeast extract broth containing 4% (vol/vol) Fildes' digest (PYF broth) (40) was used for the biochemical tests. Based on the results of the biochemical tests, each strain was identified using Bergey's *Manual of Systematic Bacteriology* (41) and copies of the *International Journal of Systematic and Evolutionary Microbiology.*

Analysis of 16S rDNA sequences. Approximately 1500 bp of the 16S rDNA gene of the isolated bacteria were sequenced according to methods published previously (42,43). One bacterial colony was scraped from the agar plate and suspended in 50 µl of TE buffer, then frozen at –20ºC and heated three times at 100ºC for 5 min each time (44). This raw DNA was used as a template for PCR. The PCR amplification was performed with a TAKARA PCR Thermal Cycler 480 (Takara, Japan). The PCR mixture contained 10× Ex Taq buffer (10 µL). A mixture of dNTP (2.5 mM) (8 µL), 4 µL of each of the primers (20 pM) (8F, 5'-AGA GTT TGA TCM TGG CTC AG; 15R, 5'-AAG GAG GTG ATC CAR CCG CA), 1 µL of raw template DNA and 3 U of Ex Taq (Takara). Distilled water was added to make the volume 100 µL, and 50 µL of mineral oil was then added. Initially, these mixtures were preheated for 3 min at 72ºC, then,

35 cycles of amplification in total were performed with denaturation of the template at 94ºC for 30 s, followed by annealing of the primers at 55ºC for 30 s, and extension of the primers at 72ºC for 2 min, and finally postheating for 2 min at 94ºC. The PCR products were separated by electrophoresis in agarose gels and stained with ethidium bromide (1 μL/mL). PCR products were purified using MicroSpin S-400HR columns (Amersham Biosciences) according to the manufacturer's instructions. The 16S rDNA gene sequence was determined by direct PCR sequencing using the method of Anzai *et al.* (45). The 16S rDNA gene sequences of other bacteria were available from GeneBank, EMBL, and DDBJ. Levels of sequence similarity were calculated and used to produce an unrooted phylogenetic tree with the neighbor-joining method (46). The alignment and stability of relationships were assessed by bootstrapping using CLUSTAL W (47).

Statistical analysis. A statistical analysis was performed with ANOVA followed by Bonferroni's *t*-test, and a *P* value less than 0.05 was considered to be significant.

RESULTS

Bile acid deconjugation in the cecal contents of GB mice inoculated with bacteria grown on NBGT agar plates. Feces from ex-GF mice inoculated with a human fecal dilution of 10^{-6}, in which most of the cecal bile acids were deconjugated, were collected. Serial dilutions of the feces were spread onto NBGT agar plates, and single colonies were isolated from the cultured plates. Among 24 bacterial strains isolated from the plates, eight strains, NB-1, NB-4, NB-6, NB-8, NB-11, NB-12, NB-13, and NB-14, were found to be capable of deconjugating TCA to CA by screening *in vitro*. Then, several mixtures of these bacterial cultures were introduced into GF mice. In the GB mice inoculated with strains NB-6, NB-8, NB-12, NB-13, and NB-14, which showed relatively high levels of deconjugating activity of TCA *in vitr*o, fecal bile acids were highly deconjugated. However, when we omitted NB-12 from this combination, the extent of deconjugation was significantly decreased (Fig. 2). Interestingly, when introduced by itself, strain NB-12 didn't colonize the GF intestine (data not shown). On the other hand, when three bacterial strains, NB-1, NB-4, and NB-11, were introduced into GF mice, the percentage of deconjugated bile acids in the intestine was low (Fig. 2). In all GB groups, inoculated bacteria were detected at concentrations of more than 10^{10}CFU/g feces.

Characterization of bile acid–deconjugating strains isolated from limited human flora associated mice. The five bacterial strains NB-6, NB-8, NB-12, NB-13, and NB-14 were characterized based on biochemical tests, and their phylogenetic positions were investigated based on the 16S rDNA sequence analysis

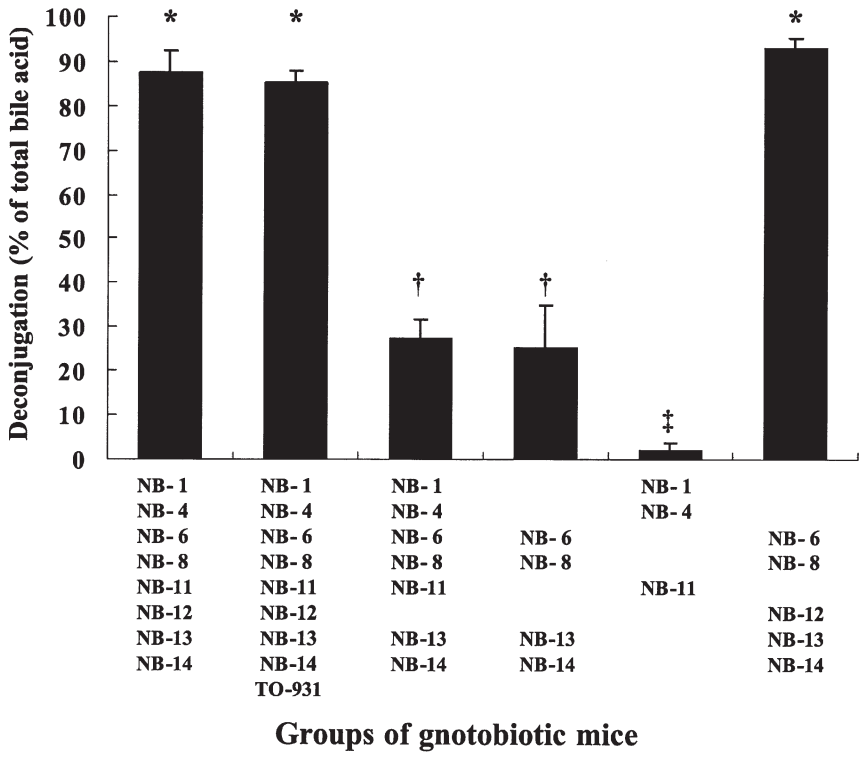

FIG. 2. Deconjugation ratio of bile acids in the cecal contents of various gnotobiotic mice associated with human intestinal bacteria. Various combinations of bacterial strains from eight bile acid–deconjugating bacteria (NB-1, NB-4, NB-6, NB-8, NB-11, NB-12, NB-13, and NB-14) and *Clostridium hiranonis* strain TO-931. NB-6. NB-8, NB-13, and NB-14 showed strong taurocholic acid-deconjugating activity and NB-1, NB-4, and NB-11 showed weak activity of deconjugation in vitro. Bars marked with different letters are significantly different ($P < 0.05$).

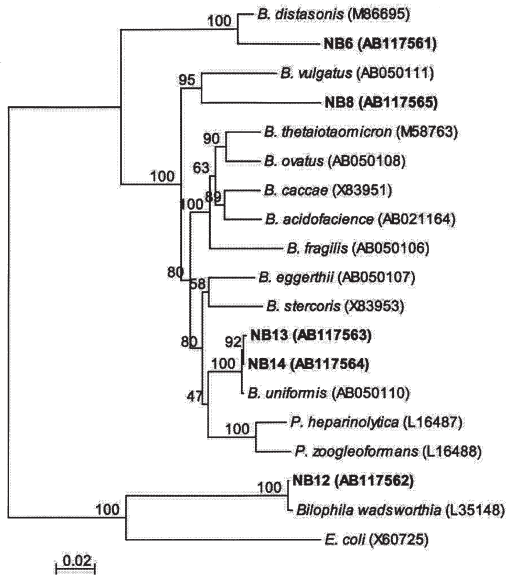

FIG. 3. Phylogenetic tree of NB strains isolated from ex–germ-free mice associated with a 10^{-6} dilution of human feces. NB strains based upon the alignment of the most similar 16S rDNA sequences with the neighbor-joining method. Bar indicates a genetic distance of 0.02.

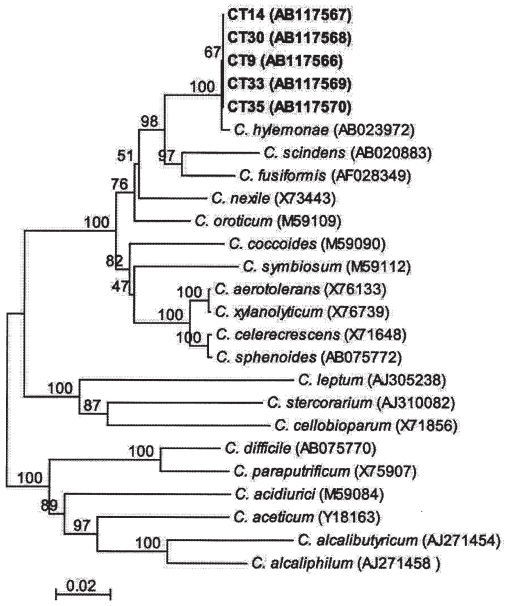

FIG. 4. Phylogenetic tree of CT strains in the *Clostridium* cluster and E. coli based upon the alignment of the most similar 16S rDNA sequences with the neighbor-joining method. Bar indicates a genetic distance of 0.02.

(Fig. 3A). NB-6 and NB-8 were identified as *Bacteroides distasonis* and *Bacteroides vulgatus*, respectively, a result confirmed by 16S rDNA sequence analysis. Strains NB-13 and NB-14 were identified as *Bacteroides uniformis*. NB-12 showed very weak responses to the regular biochemical tests using peptone-yeasts (PY) broth as a basic medium. In the sequence analysis of the 16SrDNA genes and alignment with the other sequences from the DDBJ, NB-12 showed the greatest sequence similarity with *Bilophila wadsworthia* (99.6%) (Fig. 3).

Isolation of and characterization of cholic acid 7α-dehydroxylating clostridia from limited human flora mice. The bacterial mixture from area A of each of five plates showed 100% conversion of CA to DCA. Three out of five bacterial mixtures from area B also showed 100% conversion, and the other two mixtures showing 95% and 90% conversion, respectively. On the other hand, the bacterial mixture from area C did not show any conversion of CA to DCA, suggesting that predominant clostridia in the intestine of limited flora mice inoculated with human clostridia were not capable of 7α-dehydroxylation.

We isolated 64 colonies from area B of the EG plates inoculated with the bacterial mixture from feces of limited flora mice harboring intestinal clostridia. These colonies were divided into nine groups, each containing four or eight strains. Among them, only three groups showed conversion of CA to DCA. The individual strains were screened for 7α-dehydroxylating activity *in vitro*, and finally we isolated five strains of 7α-dehydroxylating clostridia, CT-9, CT-14, CT-30, CT-33, and CT-35. All of the five strains were similar in colony morphology, being grayish and disc-shaped on EG agar plates. Cells grown on EG agar plates presented as small gram-variable, non-motile, straight to slightly curved thin rods. The sugar fermentation patterns of these bacteria were poor and variable. However, biochemical properties were identical with those described for the type strain of *C. hylemonae* strain TN-271^T, that is, H_2S and gas were produced, indole was not produced, nitrate was not reduced, and esculin and starch were not hydrolyzed. Although all of the five CT-strains belonged to *Clostridium* Cluster 14a (48) according to the 16S rDNA sequence analysis and had the highest level of similarity with *Clostridium hylemonae* (99.9%) (Fig. 4), these five strains were divided into two groups based on the production of organic acid from glucose as a source. Strains CT-30 and CT-33 produced lactate from EG broth, whereas the other strains did not.

Formation of DCA in the intestine of GB mice inoculated with a combination of isolated bacteria. DCA was detected in the feces of ex-GF mice associated with a human fecal dilution of 10^{-6}. However, we could not isolate 7α-dehydroxylating bacteria from the predominant population in the feces. The bile acid–deconjugating bacteria together with known 7α-dehydroxylating bacteria, specifically *Clostridium hiranonis* TO-931^T or *Eubacterium lentum*–like c-25, showed the formation of a small amount of DCA in the intestine of GB mice (Table 1). Further, combinations of five NB strains and five CT strains were inoculated into GF mice, and the composition of bile acid in the intestine was investigated. DCA was detected in the cecal contents of the GB mice with the five NB strains and five CT strains, equivalent to that in the cecal contents of GB mice with the five NB strains and strain TO-931^T (Table 1).

DISCUSSION

The transformation of conjugated primary bile acids into secondary bile acids by intestinal bacteria has been well investi-

TABLE 1
Composition of Cecal Bile Acid from Gnotobiotic Mice Associated with Human Intestinal Bacteria

	Groups of gnotobiotic mice					
	1*	2†	3‡	4§	5**	6††
Free form						
F-cholic acid	25.8 ± 5.9‡‡,a	7.9 ± 2.3b	7.7 ± 5.2b	25.6 ± 6.5a	2.2 ± 1.9b	6.5 ± 3.3b
F-deoxycholic acid	NDa	26.2 ± 2.1b	23.0 ± 8.6b	NDa	37.7 ± 10.0b	30.0 ± 6.9b
F-7-oxo-deoxycholic acid	1.2 ± 0.3	1.4 ± 0.9	Trace	ND	Trace	Trace
F-12-oxo-lithocholic acid	NDa	2.1 ± 1.0b	1.9 ± 1.1a,b	NDa	2.8 ± 1.9b	1.9 ± 0.5a,b
F-ursodeoxycholic acid	1.0 ± 0.2	Trace	Trace	Trace	Trace	Trace
F-α-muricholic acid	2.2 ± 0.4	2.1 ± 0.3	1.5 ± 0.1	2.1 ± 0.2	2.0 ± 0.2	1.8 ± 0.3
F-β-muricholic acid	61.5 ± 2.7	52.1 ± 0.5	50.1 ± 4.4	52.1 ± 1.5	47.0 ± 12.0	49.7 ± 6.6
F-Total	92.0 ± 3.7	93.2 ± 2.0	86.1 ± 6.6	81.1 ± 5.8	94.6 ± 1.8	91.9 ± 3.8
Taurine-conjugated form						
T-cholic acid	2.8 ± 1.2	3.6 ± 1.2	5.0 ± 2.1	8.0 ± 3.7	2.2 ± 1.0	3.1 ± 1.3
T-β-muricholic acid	4.8 ± 2.5a,b	2.6 ± 0.9a	8.1 ± 4.3a,b	10.3 ± 2.1b	2.6 ± 0.6a,b	4.4 ± 2.1a,b
T-Total	8.0 ± 3.7	6.8 ± 2.0	13.9 ± 6.6	18.9 ± 5.8	5.4 ± 1.8	8.2 ± 3.8

F-ω-muricholic acid, F-hyodeoxycholic acid, F-hyocholic acid, T-lithocholic acid, T-α-muricholic acid were not detected in all groups.
F-chenodeoxycholic acid, F-lithocholic acid, T-deoxycholic acid, T-chenodeoxycholic acid, T-ursodeoxycholic acid, were detected in trace level ND, not detected; trace, less than 1.0%.
*Gnotobiotic mice associated with eight taurocholic acid deconjugating strains,NB-1, NB-4, NB-6, NB-8, NB-11, NB-12, NB-13 and NB-14.
†Gnotobiotic mice associated with eight strains of taurocholic acid deconjugating strains plus 7α-dehydroxylating bacteria, *Clostridium hiranonis* TO-931^T.
‡Gnotobiotic mice associated with eight NB-strains of taurocholic acid deconjugating bacteria plus five CT-strains of 7α-dehydroxylating clostridia of human origin.
§Gnotobiotic mice associated with five NB-strains of taurocholic acid deconjugating bacteria, NB-6, NB-8, NB-12, NB-13 and NB-14.
**Gnotobiotic mice associated with five NB-strains and *C. hiranonis* TO-931^T.
††Gnotobiotic mice associated with five NB-strains and five CT-strains of human origin.
‡‡% of total cecal bile acids (mean ± S.D., $n = 3$).
a,bValues with different superscripts within the same column are significantly different ($P < 0.05$).

gated mostly *in vitro*. Although there are many reports of 7α-dehydroxylating bacteria, it has not been proven that such bacteria play an important part in bile acid transformation in the intestine. In rats, Uchida *et al.* reported that secondary bile acids are formed when GF rats are inoculated with clostridia of rat origin (49). However, in humans it is difficult to verify whether bacterial strains that have 7α-dehydroxylating activity *in vitro* also play an important role in bile acid formation in the colon. In this study, we isolated human fecal bacteria with the ability of transformation of bile acids in vitro and inoculated them into GF mice, and finally established GB mice with certain amount of DCA, 7α-dehydroxylated form of CA in their intestine. This result clearly indicates that the isolated bacteria from human feces could contribute secondary bile acid formation *in vivo*.

Batta *et al.* (50) reported that the deconjugation of bile acids is required for further bacterial 7α-dehydroxylation *in vitro*. We therefore attempted to use a combination of two kinds of bacterial groups, one group responsible for the deconjugation of conjugated bile acids, and the other for the 7α-dehydroxylation of primary bile acids. *Bacteroides* is known to be the predominant anaerobe in both human and mouse intestine. Notably, the "*fragilis*" group of *Bacteroides* showed growth stimulation in bile, and some selective media for *Bacteroides* contain high concentrations of sodium taurocholate (33). In the present study, GB mice inoculated with various combinations of bile acid-deconjugating strains had total bacterial counts in the feces of more than 10^{11} CFU/g feces. On the other hand, clostridial count in GB mice was 10^{10} CFU/g feces or less. These results suggest that *Bacteroides* (mainly the "*fragilis*" group) of human intestine are able to colonize in mouse intestine, and that clostridial strains in relatively low numbers are able to transform CA to DCA, if these primary bile acids are deconjugated by another bacterial group.

Strain NB-12 was identified as *Bilophila wadsworthia* based on the 16S rDNA sequence. *Bilophila wadsworthia* is reported to be isolated not only from gangrenous and perforated appendicitis, but also from normal fecal specimens (13). This strain is also bile-resistant. It is speculated that the resistance against bile or growth stimulation by bile might be related to the ability to deconjugate conjugated bile acids as reported in *Lactobacillus* or *Bifidobacterium* (10,51). Interestingly, strain NB-12 could not colonize the mouse intestine when inoculated alone. The rate of deconjugation in GB mice was lower than 30% without strain NB-12. It is suspected that a complex combination of intestinal bacteria or some metabolic or nutritional support from other bacteria is necessary to colonize the intestine.

Previously, we tried to isolate 7α-dehydroxylating bacteria from the predominant group of limited-flora mice with human intestinal clostridia. However, all the strains isolated from the predominant population, that is, bacteria grown from a 10^{-8} dilution of their feces, were negative for cholic acid 7α-dehydroxylation (31). In the present study, we used the method of Takamine and Imamura (28), which enabled us to isolate 7α-dehydroxylating bacteria in relatively small numbers in the fecal suspension.

Recently, several important strains with bile acid 7α-dehydroxylating activity were shown to belong to new phylogenetically proposed species. *Clostridium hiranonis* (52) includes strains HD-17 and TO-931^T, both of which have a high level of bile acid 7α-dehydroxylating activity (53,54). Another newly proposed species is *Clostridium hylemonae* (55), which has relatively low activity for 7α-dehydroxylation. Furthermore, the strain formerly known as *Eubacterium* sp. VPI 12708 (56) was recognized as a member of *Clostridium scindens* (55). This strain is known to be capable of 7α-dehydroxylation *in vitro,* and it is reported that a multistep biochemical pathway is needed for its transformation. The genes encoding several enzymes that either transport bile acids or catalyze various reactions in the 7α-dehydroxylation pathway of strain VPI 12708 were cloned (57). These studies strongly support the hypothesis that clostridial strains are responsible for secondary bile acid formation in the human intestine. It is reported that *Clostridium hiranonis* and *Clostridium scindens*, which have high bile acid 7α-dehydroxylation activity, could be detected by nested PCR in human fecal samples, indicating that populations of these strains are small in the intestine (58).

In this study, we isolated five strains of clostridia and found them to be most similar to *Clostridium hylemonae* based on 16SrDNA sequences. These bacteria showed 7α-dehydroxylation of CA to DCA in the GB mouse intestine when they were inoculated in combination with bile acid– deconjugating bacteria. It is strongly suggested that clostridia play an important role in transforming primary bile acids into secondary bile acids in the intestine, although the population of these bacteria in the gut is small.

Our results indicate that *Bacteroides* in human feces is one of the main bacterial groups for the deconjugation of bile acids, and clostridia play an important role in the 7α-dehydroxylation of free-form primary bile acids in the intestine. To our knowledge, this is the first report of a gnotobiotic animal model with human intestinal bacteria that can convert TCA to DCA.

Using gnotobiotic techniques, it is possible to simplify the complicated ecosystem of the intestine, and to understand the interaction between defined bacteria and the host animal. Although we should be aware that there is an obvious difference between humans and animal models, the gnotobiotic mice produced in this study could be used to study bile acid metabolism by human intestinal bacteria *in vivo,* and may provide information on regulating the metabolism by controlling these bacteria in the human intestine.

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Assignment of *Eubacterium* sp. VPI 12708 and Related Strains with High Bile Acid 7-Alpha-Dehydroxylating Activity to *Clostridium scindens* and Proposal of *Clostridium hylemonae* sp. nov., Isolated from Human Faeces, *Int. J. Syst. Evol. Microbiol. 50 Pt 3,* 971–978.

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Biomarker Validation of a Long-Chain Omega-3 Polyunsaturated Fatty Acid Food Frequency Questionnaire

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ABSTRACT: Long-chain omega-3 PUFA (LC n-3 PUFA) are beneficial for health. To date there is no specific food frequency questionnaire (FFQ) to assess LC n-3 PUFA intakes. The objective of this study is to validate our newly developed FFQ by comparison with LC n-3 PUFA content of both red blood cells (RBC) and plasma, expressed as a percentage of total FA. Fifty-three healthy male and female subjects were recruited from Wollongong, Australia. Average LC n-3 PUFA intakes (mg/d) were estimated using the new FFQ. RBC and plasma FA were assessed using GC. Spearman correlation coefficients were used to assess the linear relationship between FFQ intakes and both RBC and plasma FA. The results show that there were significant Spearman's correlation coefficients between the FFQ intakes and RBC (and plasma) FA for total LC n-3 PUFA, EPA, and DHA (0.50 (0.54), 0.39 (0.54) and 0.40 (0.48), respectively) but not for docosapentaenoic acid. The FFQ was also an effective ranking tool. The FFQ is a valid method based on erythrocyte and plasma FA as biochemical markers. In conclusion, the new FFQ is a valid method that can be used to estimate the LC n-3 PUFA intake of adults.

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The various health benefits of consuming the long-chain n-3 PUFA (LC n-3 PUFA), particularly EPA and DHA, have been reported widely (1–6). The LC n-3 PUFA are obtained predominately from fish, seafood, meat, and eggs (7,8) and in recent years from enriched food products such as bread, milk, margarine, and eggs. Recommendations for dietary intakes of LC n-3 PUFA vary considerably from the consumption of two fish meals a week (9) to EPA plus DHA intakes of 500 mg/d (10); the Japanese recommend consumption of LC n-3 PUFA of 1.6 g/d (11).

Currently there is no tailor-made food frequency questionnaire (FFQ) to assess LC n-3 PUFA intakes. We have developed a new FFQ that estimates the dietary intakes of LC n-3 PUFA. The FFQ was found to be highly reproducible and valid compared with 3-d weighed food records in 53 healthy adults (12). However, as biochemical measurements of specific nutrients in the blood or other tissues can also provide useful assessments of nutrient intake (13), it is desirable to validate the FFQ further using biomarkers of LC n-3 PUFA intake.

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Abbreviations: FFQ, food frequency questionnaire; FR, –food record; ISSFAL, International Society for the Study of Fatty Acids and Lipids; LC n-3 PUFA, long-chain n-3 PUFA; NNS, National Nutrition Survey; RBC, red blood cells.

There are several choices of a biomarker for the measurement of LC n-3 PUFA, including FA in adipose tissue (14), red blood cells (RBC), platelets, plasma, cholesterol esters, phospholipids (13), and potentially cheek cells (15). RBC may be a useful marker as they can provide an indication of the previous 120 d intake of LC n-3 PUFA, the approximate lifespan of a single RBC (16). RBC were found to be a good biomarker of LC n-3 PUFA intake as EPA and DHA were incorporated into RBC according to dose (17). Plasma has also been found to reflect LC n-3 PUFA intake in various studies (18,19).

The aims of this study were to (1) compare estimates of dietary LC n-3 PUFA intakes using the new FFQ with previously published results; (2) compare estimates of dietary LC n-3 PUFA intakes using the new FFQ with RBC FA; (3) compare estimates of dietary LC n-3 PUFA intakes with plasma FA; and (4) determine the efficacy of the FFQ to rank individuals into quintiles of intakes of LC n-3 PUFA by comparison with quintiles of RBC and plasma FA.

MATERIALS AND METHODS

Subject recruitment and clinic visits. Poster and e-mail advertisements were used to recruit staff and students from the University of Wollongong, Australia. There were no exclusion criteria, and the only requirement was that subjects had maintained a constant diet over the previous 3 mon. Vegetarians were encouraged to volunteer to ensure a wide range of dietary habits among the subjects. Based on RBC data from a previously unpublished study, it was determined that a minimum of 50 subjects should be sufficient to detect an effect size of 1 with more than 99% power at a significance level of 0.05, assuming that the correlation between methods is 0.6. The effect size is the difference between the means of the two methods, divided by the SD of either method.

Subjects attended a single clinic appointment and came in after an overnight fast to provide a fasted blood sample for RBC and plasma FA analysis. Digital scales and a wall-mounted stadiometer were used to measure weights and heights, respectively. Subjects then completed the FFQ. Approval to conduct the study was obtained from the University of Wollongong Human Research Ethics Committee, and subjects gave their written consent.

LC n-3 PUFA FFQ and determination of intakes. The 28-item semi-quantitative FFQ consists of a list of food items that are currently the only major contributors to LC n-3 PUFA intake. The foods included in the 28 questions are from the following

food groups: fish and seafood (6 questions), meats (12), cereals (4), fat spreads (2), dairy (2), and eggs (2), including LC n-3 PUFA–fortified products such as breads, milks, margarines, and eggs. Fish oil capsules are also included in the questionnaire. The FFQ also asks the subject to estimate usual meat serving size in terms of pictures of meat servings on a plate. The dietary validation of this FFQ has been published elsewhere (12). The FFQ was self-administered by subjects and took approximately 15 min to complete. The LC n-3 PUFA intakes of FFQ and the 3-d weighed Food Record (FR) were determined as described by Sullivan *et al.* (12).

Comparison with other published intakes. To determine whether the FFQ is measuring a plausible intake, another dietary intake comparator was necessary. Therefore, the intakes estimated using the FFQ were compared with those of a larger Australian sample using a 24-h recall method taken from the 1995 National Nutrition Survey (NNS) to estimate LC n-3 PUFA intake (20). The NNS collected data from 10,591 Australians aged 19 years and older, but at a time when omega-3 fortified foods were not on the market. The Wilcoxon signed rank test was used to compare the FFQ data (without the inclusion of fortified foods or fish oil capsules) to the 3-d weighed food record (FR) as previously described (12) with comparison with the NNS data (21).

Blood collection and preparation and analysis of RBC and plasma FA. Blood was collected into tubes containing EDTA and placed on ice. The samples were separated by centrifugation (10 min, 3,000 rpm, 4°C). The plasma was separated from the sample into labeled eppendorf tubes. The white buffy layer was removed and discarded, and the remaining RBC were transferred into labeled eppendorf tubes. RBC and plasma samples were stored at –80°C until analyzed.

RBC membranes were prepared for FA analysis as described by Ridges *et al.* (22). The total volume of the resuspended pellets was transferred into glass tubes for direct transesterification as described by Lepage and Roy (23). Briefly, 2 mL of methanol:toluene (4:1) was added to each sample. While vortexing, 200 μL of acetyl chloride was added dropwise to each sample using a positive displacement pipette. Samples were then heated for 60 min at 100°C in a heating block. After the tubes had been cooled in cold water, 3 mL of potassium chloride (10%) and 100 μL of toluene were added to each tube before centrifugation for 10 min (3,000 rpm, 4°C). The FAME, contained in the upper toluene phase, were removed and placed into GC vials. The FAME were analysed by flame-ionization GC (model GC-17A, Shimadzu) using a 30 m × 0.25 mm i.d. capillary column. Individual FA were identified upon comparison with known FA standards (Supelco FAME mix C4–C24 (plus added DPA), #18919-1AMP, Sigma-Aldrich, Castle Hill, Australia).

Total plasma FA were determined using the method of Lepage and Roy (23). Aliquots of 200 μL of plasma were transferred into glass tubes for direct transesterification (23) as briefly described above; however, instead of using 3 mL potassium chloride (10%), 5 mL potassium chloride (5%) was used, and instead of adding 100 μL toluene, no toluene was added. FAME were analyzed as described above.

Comparison of FFQ and LCn-3 PUFA content of RBC and plasma. Means (SD) were calculated for the total LCn-3 PUFA, EPA, docosapentaenoic acid (DPA), and DHA content (as a percentage of total FA) of the RBC and plasma. Spearman's correlation coefficients were determined for the relationship between FFQ estimated daily intakes and LCn-3 PUFA content of both RBC and plasma.

Quintile agreement. Subjects were ranked in ascending order of their daily total intake of LC n-3 PUFA estimated from the FFQ and both RBC and plasma FA. They were then separated into quintiles. Quintiles were determined by assessing the actual intakes from 53 subjects ranging from 0 to 1,100 mg/d and then dividing the subjects into 5 equal groups, where quintile 1 equals 0–98 mg/d, quintile 2 equals 99–188 mg/d, quintile 3 equals 189–304 mg/d, quintile 4 equals 305–449 mg/d, and quintile 5 equals 450–1,100 mg/d. The percentage agreement was determined between the quintile assignments using the FFQ and RBC FA, and separately for the FFQ and plasma FA.

Statistical analysis. Means (SD) were calculated for the total LCn-3 PUFA, EPA, DPA, and DHA content (as a percentage of total FA) of the RBC and plasma. The relationships between FFQ estimated daily intakes and LCn-3 PUFA content of both RBC and plasma were assessed by Spearman's correlation coefficients.

Quintile agreement was assessed by determining the percentage agreement between quintile assignments using the FFQ and RBC FA and for FFQ and plasma FA.

Statistical analysis was performed using JMP statistical analysis program (version 5.1, SAS Institute, Cary, NC). Statistical significance was set at $\alpha = 0.05$ for all analyses.

RESULTS

Fifty-three subjects (20 male and 33 female) were recruited for the validity study, including seven self-reported vegetarians. Two vegetarians were vegans, one ate extremely small amounts of fish, and two were lactoovovegetarians. The remaining two reported eating significant amounts of fish in the FFQ. The subject characteristics are presented in Table 1.

Comparison of our FFQ LC n-3 PUFA intakes with the NNS of Australia. Table 2 shows the LC n-3 PUFA intake data estimated from the FFQ (excluding fortified foods and fish oil capsules) in comparison with the analysis of the NNS of Australia conducted by Howe *et al.* (20). There were no significant differences between the estimates in this study using the FFQ and the NNS data.

Comparison of our LC n-3 PUFA FFQ with the FA content of RBC and plasma. Comparison of the LC n-3 PUFA intakes estimated from the FFQ with the percentage of the total FA content of RBC and plasma is shown in Table 3.

Bivariate plots of the FFQ estimation of LC n-3 PUFA intakes and percentage of total FA content of the RBC showed that there were good, significant relationships for total LC n-3 PUFA, EPA, and DHA (Figs. 1a, 1b, and 1d). There was no apparent relationship for DPA (Fig. 1c). Similar patterns were ob-

TABLE 1
Subject Characteristics

Subject Characteristic	Female (n = 33)	Male (n = 20)	P value
Age (years)	32 ± 11	38 ± 12	0.0670
(range)	(19–55)	(24–58)	
Height (cm)	163 ± 8	178 ± 5	<0.0001
(range)	(145–174)	(168–187)	
Weight (kg)	62 ± 11	79 ± 10	<0.0001
(range)	(43–90)	(60–100)	
BMI (kg/m^2)[a]	23 ± 3	25 ± 3	<0.05
(range)	(19–31)	(20–33)	

Values are expressed as mean ± SD.
[a]BMI, body mass index.

TABLE 2
Comparison of FFQ (n = 45) and FR (n = 45) Average Daily Intakes (mg/d) of the Individual and Total LC n-3 PUFA Without the Inclusion of Fortified Foods or Fish Oil Capsules, and Comparison with the NNS Intakes (n = 10,851).

FA	FFQ intakes (mg/d)[a]	FR intakes (mg/d)[a]	Wilcoxon signed rank test	NNS 1995 intakes (ref. 20)
Total LCn-3 PUFA	214	214	0.635	246
	(203)	(213)		
EPA	61	61	0.755	75
	(59)	(66)		
DPA	45	42	0.405	71
	(41)	(38)		
DHA	109	111	0.544	100
	(118)	(139)		

[a]Expressed as mean (SD).

served for the relationship between the FFQ estimated intakes and LC n-3 PUFA content of plasma. As seen with the RBC, there was no relationship between FFQ estimation of DPA intakes and the content of plasma as indicated by the non-significant Spearman's correlation of 0.09.

The efficacy of the FFQ to rank individuals into quintiles of intakes of LC n-3 PUFA. Subjects were also assigned quintiles based on total LC n-3 PUFA content of the RBC (% of total FA) (Table 4). Forty percent of subjects were correctly assigned into the same quintiles, and 75% of subjects were assigned to either the same or adjacent quintile. The FFQ was most successful at identifying individuals with very low intakes. Similar results were obtained for agreement of quintile assignment between FFQ total LC n-3 PUFA intakes and total LC n-3 PUFA content of plasma (Table 4). Five out of the seven vegetarian subjects were found in the first quintile for both analyses.

TABLE 3
Relationship Between Biomarker FA and Intakes from the FFQ (n = 53).

FA	RBC FA[a,b]	Plasma FA[a,b]	FFQ intakes[a]	Spearman correlation coefficient RBC vs. FFQ	Spearman correlation coefficient plasma vs. FFQ
Total LC	6.57	3.35	259	0.50[c]	0.54[c]
n-3 PUFA	(1.27)	(1.07)	(209)		
EPA	0.43	0.84	73	0.40[d]	0.54[c]
	(0.19)	(0.42)	(63)		
DPA	2.08	0.48	52	0.05	0.09
	(0.41)	(0.11)	(41)		
DHA	4.06	2.02	134	0.39[d]	0.48[c]
	(1.12)	(0.71)	(120)		

[a]Mean (SD).
[b]Expressed as % of total FA.
[c]Significant at $P < 0.001$.
[d]Significant at $P < 0.01$.

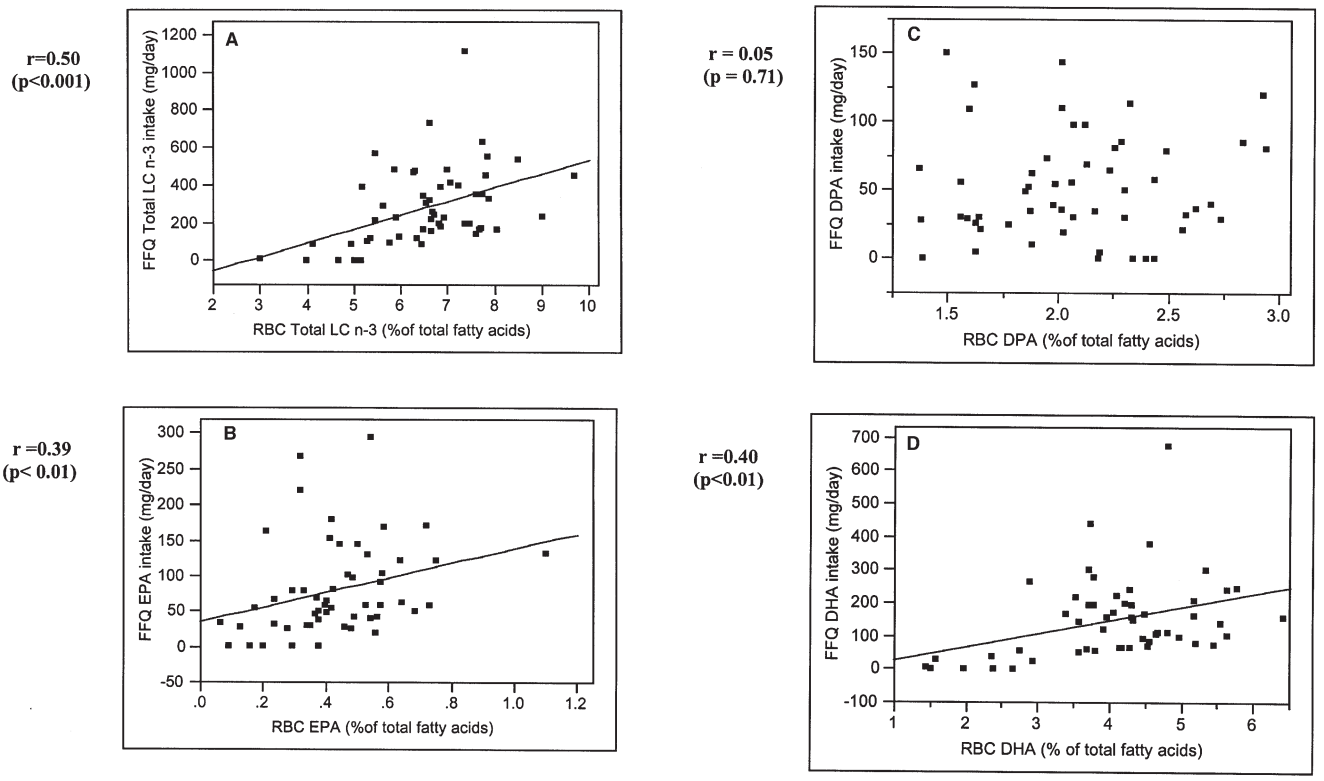

FIG. 1. (A) The relationship between estimated total LC n-3 PUFA intake from the FFQ and the LC n-3 PUFA content of the RBC with calculated Spearman correlation coefficient. (B) Bivariate plot of EPA intakes estimated by the FFQ vs. the EPA content of the RBC as a percentage of total FA with calculated Spearman correlation coefficient. (C) Bivariate plot of DPA intakes estimated by the FFQ vs. the DPA content of the RBC as a percentage of total FA with calculated Spearman correlation coefficient. (D) Bivariate plot of DHA intakes estimated by the FFQ vs. the DHA content of the RBC as a percentage of total F\A with calculated Spearman correlation coefficient.

DISCUSSION

Our new FFQ estimates the dietary intakes of LC n-3 PUFA very well as the results compare favorably with the results from the NNS of Australia (Table 2, and ref. 20). The NNS intakes of EPA and DPA were slightly higher and the NNS intakes of DHA were slightly lower than in our study, which could be explained by the fact that our study group contained seven vegetarians. With meat being a rich source of DPA (24), the inclusion of seven vegetarian subjects (who did not eat meat) in this study may account for the lower DPA intakes.

Other studies have used various biomarkers to validate newly developed or existing FFQ, although not many have been validated for LC n-3 PUFA intake, and those that do use biomarkers other than RBC. Woods *et al.* (25) validated an existing semiquantitative FFQ for measuring fish intake with plasma FA composition. That FFQ was assessing overall dietary intake over the preceding 12 mon and was originally developed for use by the Anti Cancer Council of Victoria. Significant Pearson correlation coefficients were obtained between percentage of plasma FA and grams of steamed, grilled, or baked fish and grams of total non-fried fish consumed ($r = 0.33$

TABLE 4
Agreement of Quintile Assignment Between FFQ Total LC n-3 PUFA Intakes and Total LC n-3 PUFA Content of Both RBC and Plasma ($n = 53$)

	FFQ intakes vs. RBC			FFQ intakes vs. plasma		
Quintile	Same quintile	Adjacent quintile	Incorrectly classified	Same quintile	Adjacent quintile	Incorrectly classified
1 ($n = 10$)	8	1	1	7	2	1
2 ($n = 11$)	2	5	4	2	5	4
3 ($n = 11$)	5	5	1	3	6	2
4 ($n = 11$)	3	4	4	1	8	2
5 ($n = 10$)	3	4	3	2	5	3
Total no. (%)	21 (40)	19 (36)	13 (24)	15 (28)	26 (49)	12 (23)

for DHA and 0.34 for total LC n-3 PUFA). Significant Pearson correlation coefficients were also obtained between the percentage of total plasma n-3 PUFA and the same two variables (both 0.33). Our FFQ shows higher Spearman's correlation coefficients ($r = 0.50$ for total LC n-3 PUFA, $r = 0.39$ for EPA, and $r = 0.40$ for DHA), hence our newly developed FFQ is an improvement on previously validated FFQ to estimate LC n-3 PUFA intakes (25).

There are a few reasons why our new FFQ shows higher correlations. First, in Woods *et al.* (25), intakes of fish (steamed, baked, grilled, fried, and takeaway fish) and tinned fish were calculated without the inclusion of other types of seafood. There was no distinction between different fish species, which are known to have large variations in LC n-3 PUFA content (24). Second, other foods that contain LC n-3 PUFA, such as eggs and meat, were not included in the analysis (8), and these may account for nearly 50% of LC n-3 PUFA intakes (20). Finally, a major limitation was that at the time of the study there was no Australian database for the estimation of DHA and EPA intakes from the FFQ, which has now been rectified by Mann *et al.* (26).

Our correlation coefficients between RBC and dietary intakes of LC n-3 PUFA compare favorably with the study by Anderson *et al.* (18). Correlation coefficients between plasma phospholipid FA and dietary intakes of EPA and DHA were 0.51 and 0.49, respectively. Their correlation between fish intake and n-3 PUFA in plasma phospholipids was 0.37, whereas our correlation coefficient of total intake of LC n-3 PUFA and RBC LC n-3 PUFA was 0.5. Their lower correlation between fish intake and n-3 PUFA in plasma phospholipids further highlights the fact that foods other than fish and seafood contribute to LC n-3 PUFA intake (20).

The mean percentages of LC n-3 PUFA in RBC in our study were comparable to those determined in other studies (17,26). LC n-3 PUFA content of plasma was, however, lower than reported in other studies (25,27). RBC and plasma were found to be good biomarkers of total LC n-3 PUFA, EPA, and DHA intakes as highlighted by the significant Spearman's correlation coefficients, but RBC and plasma were not found to be good biomarkers of DPA intakes ($r = 0.05$). Significant correlations between dietary intakes and RBC FA were expected for EPA and DHA as both FA have been found to be incorporated into RBC by dose (17). However, DPA intakes estimated from the FFQ were found to be highly reproducible with a highly significant Spearman's correlation coefficient of 0.89 (12). This evidence strongly suggests that the lack of correlation is not due to the FFQ instrument itself and that it is more likely due to biological or metabolic factors and this warrants further investigation.

Quintile assignment proved that the FFQ was able to adequately identify individuals according to their RBC levels of LC n-3 PUFA. Five out of the seven vegetarians were correctly assigned to the first (lowest intake) quintile based on intakes measured by the FFQ and LC n-3 content of the RBC. Agren *et al.* (28) also found that Finnish vegans had significantly lower EPA, DPA, and DHA in their RBC than omnivorous subjects. The other two self-reported vegetarians, as expected, had higher LC n-3 PUFA content of RBC due to significant intakes of fish and seafood. Overall, the quintile assignment in this study was better than or consistent with other studies. The FFQ used by Erkkola *et al.* (29) classified 62% of individuals into the same or adjacent quintiles based on total n-3 PUFA intakes. The ability of this FFQ to classify more than 75% of individuals into identical or adjacent quintiles may be useful in clinical settings to identify individuals with low LC n-3 PUFA status.

In summary, the FFQ performed well against the RBC membrane and total plasma FA, apart from the lack of correlation between DPA intakes and DPA content of both RBC and plasma, suggesting that these may not be good biomarkers of DPA intake. The FFQ is therefore an adequate dietary assessment tool for the estimation of total LC n-3 intakes in healthy Australian adults and would be a useful screening tool for low intakes of LC n-3 PUFA. It is an appealing alternative to other dietary assessment methods due to its validity, low subject burden and low cost, and being very fast to complete. In conclusion, the FFQ is a valid dietary assessment tool for the estimation of dietary LC n-3 PUFA.

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CODE: _____

(office use only)

This questionnaire is available for use for research purposes only. Due reference should be made to our publication in *Lipids* (Sullivan *et al.,* *Lipids 41*, 845–850, 2006). If you are using this questionnaire to determine the long-chain omega-3 PUFA intakes in Australia, then contact Dr. Barbara Meyer (bmeyer@uow.edu.au or phone 02 4221 3459) to obtain further information regarding the automated electronic version of this questionnaire.

The Long-Chain Omega-3 Polyunsaturated Fatty Acid Questionnaire

INSTRUCTIONS:

This questionnaire consists of 28 questions about your **usual** eating habits **over the past 12 months**.

Before completing the questionnaire, please fill in the following information:

The date that you completed this questionnaire: _____

Your gender: ☐ male ☐ female

Your age: ☐ under 19 ☐ 19-24 ☐ 25-29 ☐ 30-34 ☐ 35-39 ☐ 40-44
☐ 45-49 ☐ 50-54 ☐ 55-59 ☐ 60-64 ☐ 65 and over

Human Milk Fatty Acid Composition from Nine Countries Varies Most in DHA

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ABSTRACT: Many published studies of breast milk FA composition are limited to populations from one or two countries. We aimed to examine the degree to which FA compositions vary across a number of diverse populations. Because diet and maternal adipose stores influence breast milk FA composition, differences in FA composition between groups most likely reflect habitual dietary differences. Approximately 50 breast milk samples (full breast expression) were collected from women in Australia, Canada, Chile, China, Japan, Mexico, Philippines, the United Kingdom, and the United States. The proportion of saturated FA was relatively constant among countries, with the exception of the Philippines, where levels of lauric and myristic acids were elevated (means greater than two times the mean of most other countries). Monounsaturated FA also varied little, with the exception of low levels of oleic acid in the Philippines and high levels of erucic acid in China. Although arachidonic acid (C20:4n-6) levels were similar among all countries (means ranging from 0.36 wt % to 0.49 wt %), mean DHA (C22:6n-3) levels ranged from 0.17 to 0.99 wt %, with the highest levels in Japanese milk and the lowest levels in Canadian and U.S. samples. The results of this study demonstrate that the proportion of saturated and monounsaturated FA are relatively constant across a large number of countries, whereas the level of some of the PUFA, especially DHA, are highly variable.

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Breast feeding is the most appropriate means of providing nutrition to healthy term infants. Human milk is not a static, invariant fluid, but fluctuates in micro- and macronutrient composition over the course of a feeding, longitudinally from birth to weaning, and, at least for some components, as a consequence of maternal diet. For example, during the course of a single feed, total lipid concentration increases several fold from foremilk to hindmilk (1). Total nitrogen content for human milk falls precipitously during the first month of lactation, but does not change dramatically after that (2). Diet affects levels of fat-soluble components of human milk, such as fatty acids (FA), carotenoids, vitamin A, and vitamin E (3–5).

The lipids of human milk provide approximately half of the energy needs for the neonate, and also provide essential FA and fat-soluble vitamins. The levels of some FA in human milk, such as docosahexaenoic acid (DHA, 22:6n3), are clearly dependent on dietary intake. Women who consume fish and other foods containing high levels of n–3 long-chain PUFA (LCP) have relatively high milk DHA levels compared with milk from women who consume diets low in these components (6,7). Milk DHA levels increase in a dose-related manner in women receiving either fish oil supplements (8) or DHA supplements from an algal single cell oil (9). Linoleic acid (LA, C18:2n-6) in breast milk is also dependent on diet. The consumption of PUFA has increased in many countries over the past 40 years, with the result that the proportion of LA in human milk has doubled in countries such as the United States (10). Beyond specific dietary FA, total maternal dietary fat intake also alters the concentration of selected human milk FA. For example, levels of lauric acid (C12:0) are relatively low in the milk of women who consume moderate- to high-fat diets; however, in women consuming low-fat diets, mammary gland FA synthesis is elevated, which increases the level of milk lauric acid (11–13).

The present study was designed to evaluate the FA profiles of a large number of mature (postpartum day ≥ 30) human milk samples from each of nine countries. The collection countries were designed to be reflective of a global sample with collection sites in Asia (China, Japan, and the Philippines), Australia, Europe (the United Kingdom), North America (Canada, the United States, and Mexico), and South America (Chile).

MATERIALS AND METHODS

The study subjects were healthy, nonsmoking mothers (age 14 to 41 y), exclusively breastfeeding single-birth, full-term, healthy infants aged 1 to 12 mon. Mothers were under no rigorous dietary restrictions and their diets included at least three servings of fruits and vegetables per day. Mothers recorded their 24-h dietary intake records on the day prior to their milk donation, and all records were reviewed at study completion by one dietitian; the number of servings of fruits and vegetables for each diet record was calculated using the American Dietetic Association Exchange Lists. The pediatric clinics that participated in recruitment served middle socioeconomic populations, with the exceptions of Manila, Philippines, and Chengdu, China (lower socioeconomic class).

Milk samples represented a single full breast expression and were collected by electric pump (with the exception of Japan, where milk was collected by manual expression) between 1:00 and 5:00 PM, 2–4 h after the last feeding. Written informed

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Abbreviations: AA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; LA, linoleic acid; long-chain PUFA (LCP); PUFA, polyunsaturated fatty acid.

consent was obtained from all subjects. Following expression, milk samples were immediately placed on dry ice or in a freezer at –20°C, then shipped within 10–14 d on dry ice to a central laboratory where the samples were stored at –70°C. Lipid was extracted from the milk using a modified Roese Gottlieb mixed ethers extraction (14). Samples were acidified prior to extraction to ensure recovery of any free FA generated by lipase activity. Total lipids were determined for each milk sample and reported in a previous publication (4). Fatty acid methyl esters (FAME) were prepared with BF_3/methanol (14% wt/vol) and extracted into hexane (15).

Analyses were performed with a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector (Little Falls, DE). The carrier gas was hydrogen with a linear velocity of 45 cm/s and a 1:60 split ratio. The column was a 30-m capillary (0.32 mm i.d.) Omegawax™ (Supelco, Bellefonte, PA). Chromatographic conditions were isothermal, 200°C. The injector and detector temperatures were 220°C and 230°C, respectively. FA were identified by equivalent chain length as determined using retention times generated by reference mixes of FAME. Detector response factors were calculated using quantitative reference mixes of FAME (Nu Chek Prep, Elysian, MN).

Most of the FA were normally distributed, and normal distribution was assumed for the statistical analyses of the FA profiles. The weight percent of human milk FA for the nine countries were compared by the Student-Newman-Keuls test at the $P = 0.05$ level following a one-way ANOVA. Ratios of LA:alpha-linolenic acid (ALA, C18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3):DHA, and AA:DHA were calculated for each sample, and then the mean ratios were determined for each country. Concentrations of LA, AA, ALA, and DHA were calculated from the FA profiles and the concentration of total lipid (reported in reference 4) for each sample. These values were not corrected for lipids other than triglycerides; triglycerides account for greater than 98% of lipids in human milk (11). The concentration data were not normally distributed and therefore a log transformation was conducted prior to statistical analysis (16).

RESULTS

Study population. The total number of samples was 440, and the mean age of mothers was 29.5 y. Mean parity was 1.2. The number of participants per country varied from 44 to 54. A more complete description of the mothers is provided in a previous publication (4).

Saturated FA. Lauric acid was only a modest component of milk FA, with means of 4.24–6.15 wt % in all countries, with the exception of the Philippines, where the mean was 13.82 wt % (Table 1). Myristic acid (C14:0) followed a similar pattern (a range of 3.61% to 6.80% for all countries except the Philippines, where the level was 12.12%). For these FA, the mean level in the Philippines was significantly higher than all other countries. Palmitic acid (C16:0) was the predominant saturated FA in all countries, with Philippine samples having the highest

TABLE 1
Human Milk Saturated FA (% of Total FA)[a]

Country of origin	Australia	Canada	Chile	China	Japan
no. of women	48	48	50	50	51
C8:0	0.20 ± 0.01^{b,c}	0.17 ± 0.0^{a,b}	0.20 ± 0.0^{b,c}	0.17 ± 0.0^{a,b}	0.22 ± 0.01^{c}
C10:0	1.62 ± 0.04^{a,b}	1.66 ± 0.05^{b}	1.87 ± 0.06^{c}	1.67 ± 0.06^{b}	2.00 ± 0.05^{c}
C12:0	5.49 ± 0.22^{b,c}	5.25 ± 0.23^{a,b,c}	6.15 ± 0.34^{c}	4.24 ± 0.26^{a}	5.86 ± 0.24^{c}
C13:0	0.03 ± 0.00^{c,d}	0.04 ± 0.00^{c,d}	0.04 ± 0.00^{d}	0.01 ± 0.00^{a}	0.03 ± 0.00^{b}
C14:0	6.28 ± 0.23^{c,d}	5.84 ± 0.27^{b,c,d}	6.80 ± 0.34^{d}	3.61 ± 0.23^{a}	6.11 ± 0.25^{c,d}
C15:0	0.39 ± 0.01^{e}	0.33 ± 0.01^{c,d}	0.30 ± 0.01^{c}	0.12 ± 0.01^{a}	0.29 ± 0.01^{c}
C16:0	22.26 ± 0.31^{c}	18.67 ± 0.32^{a}	18.79 ± 0.30^{a}	18.62 ± 0.26^{a}	20.20 ± 0.25^{b}
C17:0	0.41 ± 0.01^{d}	0.32 ± 0.01^{b,c}	0.35 ± 0.01^{c}	0.22 ± 0.01^{a}	0.32 ± 0.01^{b,c}
C18:0	6.77 ± 0.20^{c}	5.83 ± 0.12^{b}	5.77 ± 0.16^{b}	6.13 ± 0.14^{b}	6.14 ± 0.16^{b}
C20:0	0.20 ± 0.01^{b,c}	0.20 ± 0.01^{b,c}	0.21 ± 0.01^{c}	0.20 ± 0.01^{b,c}	0.20 ± 0.01^{b,c}
C22:0	0.08 ± 0.00^{b}	0.10 ± 0.00^{b}	0.09 ± 0.00^{b}	0.09 ± 0.01^{b}	0.09 ± 0.00^{b}
C24:0	0.06 ± 0.00^{a}	0.06 ± 0.00^{a}	0.08 ± 0.01^{b}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}
Country of origin	Mexico	Philippines	United Kingdom	United States	
No. of women	46	54	44	49	
C8:0	0.19 ± 0.01^{b,c}	0.28 ± 0.01^{d}	0.20 ± 0.01^{b,c}	0.16 ± 0.01^{a}	
C10:0	1.46 ± 0.04^{a}	2.35 ± 0.06^{d}	1.84 ± 0.05^{c}	1.50 ± 0.05^{a,b}	
C12:0	4.97 ± 0.23^{a,b,c}	13.82 ± 0.47^{d}	4.99 ± 0.24^{a,b,c}	4.40 ± 0.22^{a,b}	
C13:0	0.03 ± 0.00^{b}	0.03 ± 0.00^{b,c}	0.05 ± 0.00^{e}	0.02 ± 0.00^{b}	
C14:0	5.57 ± 0.24^{b,c}	12.12 ± 0.39^{e}	5.87 ± 0.23^{b,c,d}	4.91 ± 0.23^{b}	
C15:0	0.32 ± 0.01^{c}	0.21 ± 0.01^{b}	0.36 ± 0.02^{d}	0.29 ± 0.01^{c}	
C16:0	19.91 ± 0.25^{b}	23.02 ± 0.28^{c}	22.59 ± 0.31^{c}	19.26 ± 0.29^{a,b}	
C17:0	0.33 ± 0.01^{c}	0.24 ± 0.01^{a}	0.29 ± 0.01^{b}	0.32 ± 0.01^{b,c}	
C18:0	6.07 ± 0.15^{b}	4.75 ± 0.13^{a}	6.25 ± 0.15^{b}	6.21 ± 0.18^{b}	
C20:0	0.18 ± 0.00^{b}	0.13 ± 0.00^{a}	0.20 ± 0.01^{b,c}	0.19 ± 0.01^{b}	
C22:0	0.08 ± 0.00^{b}	0.06 ± 0.00^{a}	0.08 ± 0.00^{b}	0.09 ± 0.01^{b}	
C24:0	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.06 ± 0.00^{a}	0.06 ± 0.00^{a}	

[a]Data are mean ± standard error. Means in the same row with different superscripts differ significantly at $P < 0.05$.

TABLE 2
Human Milk Monounsaturated FA (% of Total FA)[a]

Country of origin	Australia	Canada	Chile	China	Japan
no. of women	48	48	50	50	51
C14:1n-5	0.31 ± 0.01^{e}	0.25 ± 0.01^{c,d}	0.18 ± 0.01^{b}	0.06 ± 0.00^{a}	0.20 ± 0.01^{b}
C16:1n-9	0.42 ± 0.01^{c,d,e}	0.23 ± 0.03^{b}	0.40 ± 0.03^{c,d}	0.49 ± 0.01^{e}	0.36 ± 0.02^{c,d}
C16:1n-7	2.97 ± 0.11^{b}	2.79 ± 0.12^{b}	2.70 ± 0.10^{b}	1.88 ± 0.05^{a}	2.56 ± 0.08^{b}
C16:1n-5	0.12 ± 0.00^{d}	0.10 ± 0.00^{b,c}	0.11 ± 0.00^{c,d}	0.03 ± 0.00^{a}	0.09 ± 0.00^{b}
C16:1n-3	0.19 ± 0.01^{f}	0.12 ± 0.00^{c,d}	0.06 ± 0.00^{a,b}	0.05 ± 0.00^{a}	0.12 ± 0.00^{c,d}
C17:1n-7	0.34 ± 0.01^{f}	0.28 ± 0.01^{e}	0.27 ± 0.01^{d,e}	0.17 ± 0.00^{a}	0.25 ± 0.01^{b,c,d}
C18:1n-9	32.23 ± 0.44^{c,d,e}	35.18 ± 0.50^{f}	26.19 ± 0.46^{b}	36.49 ± 0.45^{g}	31.43 ± 0.40^{c,d}
C18:1n-7	2.37 ± 0.04^{b,c}	2.85 ± 0.05^{d,e}	2.67 ± 0.06^{d}	2.13 ± 0.04^{a}	2.32 ± 0.04^{b,c}
C18:1n-5	0.35 ± 0.02^{g}	0.26 ± 0.01^{e}	0.21 ± 0.01^{d}	0.08 ± 0.00^{a}	0.18 ± 0.01^{c}
C20:1n-11	0.23 ± 0.01^{b,c}	0.20 ± 0.01^{b}	0.23 ± 0.01^{b,c}	0.06 ± 0.00^{a}	0.27 ± 0.04^{c}
C20:1n-9	0.38 ± 0.01^{b}	0.52 ± 0.01^{c,d}	0.55 ± 0.03^{d}	1.25 ± 0.06^{e}	0.52 ± 0.02^{c,d}
C22:1n-9	0.08 ± 0.00^{a}	0.11 ± 0.00^{a}	0.14 ± 0.01^{a}	1.21 ± 0.14^{b}	0.13 ± 0.01^{a}

Country of origin	Mexico	Philippines	United Kingdom	United States
No. of women	46	54	44	49
C14:1n-5	0.20 ± 0.01^{b}	0.50 ± 0.02^{f}	0.28 ± 0.02^{d}	0.22 ± 0.01^{b,c}
C16:1n-9	0.35 ± 0.03^{c}	0.08 ± 0.02^{a}	0.44 ± 0.01^{d,e}	0.44 ± 0.01^{d,e}
C16:1n-7	2.64 ± 0.09^{b}	4.59 ± 0.15^{c}	2.85 ± 0.12^{b}	2.64 ± 0.11^{b}
C16:1n-5	0.09 ± 0.00^{b}	0.20 ± 0.01^{e}	0.11 ± 0.00^{c,d}	0.10 ± 0.00^{b,c}
C16:1n-3	0.13 ± 0.00^{d,e}	0.06 ± 0.00^{b}	0.14 ± 0.01^{e}	0.11 ± 0.00^{c}
C17:1n-7	0.24 ± 0.01^{b,c}	0.23 ± 0.01^{b}	0.27 ± 0.01^{d,e}	0.26 ± 0.01^{c,d,e}
C18:1n-9	30.79 ± 0.44^{c}	21.85 ± 0.51^{a}	33.28 ± 0.40^{e}	32.77 ± 0.48^{d,e}
C18:1n-7	2.72 ± 0.05^{d,e}	2.25 ± 0.07^{a,b}	2.50 ± 0.05^{c}	2.88 ± 0.09^{e}
C18:1n-5	0.23 ± 0.01^{d}	0.14 ± 0.01^{b}	0.18 ± 0.01^{c}	0.30 ± 0.01^{f}
C20:1n-11	0.22 ± 0.01^{b,c}	0.08 ± 0.01^{a}	0.19 ± 0.01^{b}	0.21 ± 0.01^{b,c}
C20:1n-9	0.42 ± 0.01^{b}	0.28 ± 0.01^{a}	0.44 ± 0.01^{b,c}	0.39 ± 0.01^{b}
C22:1n-9	0.08 ± 0.00^{a}	0.07 ± 0.00^{a}	0.10 ± 0.00^{a}	0.08 ± 0.00^{a}

[a]Data are mean ± standard error. Means in the same row with different superscripts differ significantly at $P < 0.05$.

level. In contrast to other saturated FA, Philippine samples had the lowest level of stearic acid (C18:0).

Monounsaturated FA. Oleic acid (C18:1n-9) was the predominant monounsaturated FA in breast milk samples from all countries (Table 2), and, with the exception of the Philippines, was also present at higher proportions than any saturated or PUFA. The oleic acid levels in most countries were above 30%, with the exception of Chile (26.19%) and the Philippines (21.85%). Erucic acid (C22:1n-9) levels were below 0.2% in all countries with the exception of China, where the mean level was 1.21%. Eicosenoic acid (C20:1n-9) followed a similar pattern to erucic acid, with the levels in China being greater than twice that of any other country (1.25% in China vs. a range of 0.28% to 0.55% in other countries).

PUFA. LA levels ranged from 7.90% in the Philippines to 17.75% in Chile (Table 3), with the mean level of all samples being 12.93%. The levels of AA were relatively constant across all countries (Table 3, Fig. 1), with the mean level of all samples being 0.41%. Other n-6 FA (18:3n-6, 20:3n-6) also fell within relatively narrow ranges. The ALA levels were constant among most countries at approximately 1% of FA (Table 4). However, the ALA levels in samples from China were significantly higher (2.02%), and levels in samples from the Philippines were significantly lower (0.43%) than all other countries. The mean ALA level of all samples was 1.14%. DHA levels were much more variable, with a range from 0.17% in the United States and Canada to 0.99% in Japan, with a global mean of 0.41% (Table 4, Fig. 2). Countries with the highest DHA levels also had the highest EPA (C20:5n-3) levels (Japan: 0.26%; the Philippines: 0.15%).

The LA:ALA ratio varied approximately twofold among countries, whereas the AA:DHA ratio varied more widely, from 0.51:1 in Japan to 3.16:1 in the United States (Table 4). The mean AA:DHA ratio for all samples was 1.63:1. The EPA:DHA ratio varied inversely with the DHA content: the EPA:DHA ratio was high in countries with low DHA (Australia, Canada, the United Kingdom, and the United States), and was low in countries with high DHA (Chile, China, Japan, and the Philippines).

The concentrations of the essential FA, LA and ALA, as well as the LCP AA and DHA, were calculated from the FA profiles and the fat concentration in each milk sample (Table 5). High total milk fat in the samples from China resulted in relatively high concentrations of all PUFA from that country.

DISCUSSION

The current study evaluated the FA profiles of approximately 50 mature human milk samples from each of nine countries, making this one of the largest analyses of its type to date. Many previous studies have reported values from one or two countries; however, methodological differences make comparisons among studies difficult. The present study employed the same collection and analysis conditions, which permit a comparison

TABLE 3
Human Milk n-6 PUFA (% of Total FA)[a]

Country of origin	Australia	Canada	Chile	China	Japan
No. of women	48	48	50	50	51
C14:2n-6	0.15 ± 0.01^{d}	0.10 ± 0.01^{b}	0.10 ± 0.01^{b}	0.04 ± 0.00^{a}	0.10 ± 0.00^{b}
C16:2n-6	0.29 ± 0.01^{d}	0.22 ± 0.01^{b,c}	0.24 ± 0.01^{c}	0.10 ± 0.00^{a}	0.20 ± 0.01^{b}
C18:2n-6	10.66 ± 0.34^{b}	11.48 ± 0.42^{b}	17.75 ± 0.58^{e}	14.88 ± 0.42^{d}	12.66 ± 0.25^{c}
cis,trans- C18:2n-6	0.09 ± 0.00^{b}	0.25 ± 0.01^{f}	0.16 ± 0.01^{d}	0.09 ± 0.00^{b}	0.12 ± 0.01^{c}
CLA c9,t11	0.28 ± 0.01^{f}	0.21 ± 0.01^{c}	0.22 ± 0.01^{c,d}	0.07 ± 0.01^{a}	0.13 ± 0.00^{b}
CLA t10,c12	0.06 ± 0.00^{d}	0.05 ± 0.00^{c,d}	0.05 ± 0.00^{b,c}	n.d	0.03 ± 0.00^{a}
C18:3n-6	0.17 ± 0.01^{d}	0.16 ± 0.01^{c,d}	0.15 ± 0.01^{b,c}	0.15 ± 0.01^{b,c}	0.13 ± 0.00^{b}
C20:2n-6	0.20 ± 0.01^{a}	0.21 ± 0.01^{a,b}	0.54 ± 0.03^{f}	0.39 ± 0.01^{e}	0.25 ± 0.01^{b,c}
C20:3n-6	0.31 ± 0.01^{b,c}	0.27 ± 0.01^{a,b}	0.44 ± 0.02^{d}	0.28 ± 0.01^{a,b}	0.25 ± 0.01^{a}
C20:4n-6	0.38 ± 0.01^{a}	0.37 ± 0.01^{a}	0.42 ± 0.02^{a,b}	0.49 ± 0.02^{c}	0.40 ± 0.01^{a}
C22:2n-6	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.08 ± 0.01^{e}	0.06 ± 0.00^{d}	0.02 ± 0.00^{a,b}
C22:4n-6	0.09 ± 0.00^{b}	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	0.11 ± 0.00^{c}	0.08 ± 0.00^{b}
C22:5n-6	0.04 ± 0.00^{a,b}	0.04 ± 0.00^{a,b}	0.09 ± 0.01^{d}	0.06 ± 0.00^{c}	0.05 ± 0.00^{a,b,c}
Total C20 + C22 n-6 series	1.03 ± 0.03^{a,b}	0.94 ± 0.02^{a}	1.61 ± 0.08^{e}	1.40 ± 0.03^{d}	1.05 ± 0.03^{a,b}

Country of origin	Mexico	Philippines	United Kingdom	United States
No. of women	46	54	44	49
C14:2n-6	0.11 ± 0.01^{b}	0.04 ± 0.00^{a}	0.13 ± 0.01^{c}	0.09 ± 0.00^{b}
C16:2n-6	0.23 ± 0.01^{c}	0.09 ± 0.00^{a}	0.22 ± 0.01^{b,c}	0.20 ± 0.01^{b}
C18:2n-6	16.05 ± 0.51^{d}	7.90 ± 0.24^{a}	10.45 ± 0.41^{b}	14.78 ± 0.39^{d}
cis,trans- C18:2n-6	0.20 ± 0.01^{e}	0.06 ± 0.00^{a}	0.09 ± 0.00^{b}	0.35 ± 0.02^{g}
CLA c9,t11	0.20 ± 0.01^{c}	0.08 ± 0.00^{a}	0.26 ± 0.01^{e,f}	0.24 ± 0.01^{d,e}
CLA t10,c12	0.04 ± 0.00^{a,b}	0.03 ± 0.00^{a}	0.04 ± 0.01^{b,c}	0.06 ± 0.00^{c,d}
C18:3n-6	0.15 ± 0.00^{c,d}	0.10 ± 0.00^{a}	0.17 ± 0.00^{d}	0.17 ± 0.01^{d}
C20:2n-6	0.34 ± 0.01^{d}	0.23 ± 0.01^{a,b,c}	0.22 ± 0.01^{a,b}	0.27 ± 0.01^{c}
C20:3n-6	0.33 ± 0.01^{c}	0.31 ± 0.01^{b,c}	0.33 ± 0.01^{c}	0.35 ± 0.02^{c}
C20:4n-6	0.42 ± 0.01^{a,b}	0.39 ± 0.01^{a}	0.36 ± 0.01^{a}	0.45 ± 0.02^{b}
C22:2n-6	0.03 ± 0.00^{b}	0.05 ± 0.00^{c}	0.04 ± 0.00^{b,c}	0.06 ± 0.00^{d}
C22:4n-6	0.11 ± 0.00^{c}	0.11 ± 0.00^{c}	0.08 ± 0.00^{b}	0.11 ± 0.00^{c}
C22:5n-6	0.05 ± 0.00^{b,c}	0.08 ± 0.00^{d}	0.03 ± 0.00^{a}	0.06 ± 0.00^{b,c}
Total C20 + C22 n-6 series	1.28 ± 0.03^{c,d}	1.17 ± 0.02^{b,c}	1.07 ± 0.03^{a,b}	1.29 ± 0.04^{c,d}

[a]Data are mean ± standard error. Means in the same row with different superscripts differ significantly at $P < 0.05$. n.d., not detected.

among countries independent of methodological variations. Although the data are reported as samples coming from a specific country, it must be kept in mind that the samples from each country were taken from one specific geographical area, and are specific solely to that area, not to the country as a whole. Previous studies have demonstrated regional differences in several countries, such as France (17) and China (18).

Parallels can be seen between the levels of several milk FA and maternal diets. For example, our data demonstrated that milk LA levels were relatively high in countries typically consuming

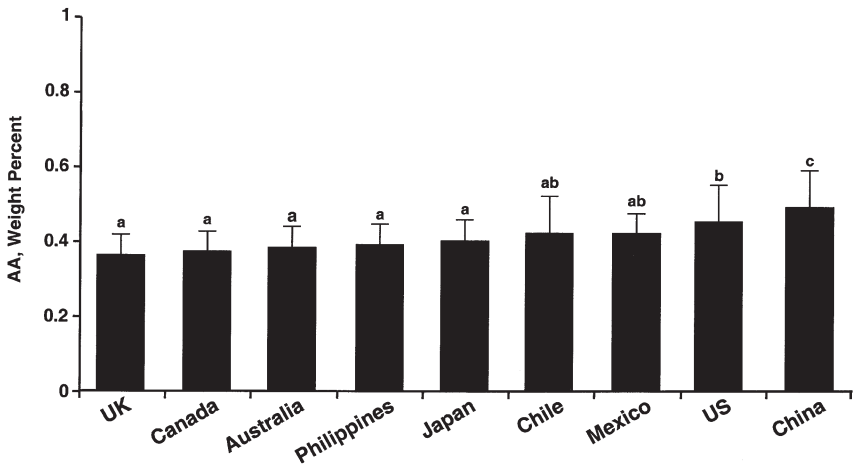

FIG. 1. AA as weight percent of total FA in mature human milk of women from nine countries. Values are means ± SEM with 44 to 54 samples per group. Means with different superscripts are statistically different ($P < 0.05$).

TABLE 4
Human Milk n-3 PUFA (% of Total FA)[a]

Country of origin	Australia	Canada	Chile	China	Japan
No. of women (% wt/wt, mean ± SE)	48	48	50	50	51
C18:3n-3	0.90 ± 0.04[b]	1.22 ± 0.05[c,d]	1.14 ± 0.07[c,d]	2.02 ± 0.06[e]	1.33 ± 0.05[d]
C18:4n-3	0.01 ± 0.00[a,b]	0.02 ± 0.00[a,b]	0.01 ± 0.00[a]	0.05 ± 0.00[c,d]	0.06 ± 0.02[d]
C20:3n-3	0.04 ± 0.00[a]	0.04 ± 0.00[a,b]	0.07 ± 0.00[c]	0.13 ± 0.00[d]	0.05 ± 0.00[b]
C20:4n-3	0.08 ± 0.00[a,b]	0.08 ± 0.00[a,b]	0.09 ± 0.00[a,b,c]	0.10 ± 0.00[b,c]	0.12 ± 0.01[d]
C20:5n-3	0.10 ± 0.01[a]	0.08 ± 0.00[a]	0.09 ± 0.01[a]	0.07 ± 0.00[a]	0.26 ± 0.02[c]
C22:5n-3	0.18 ± 0.01[b]	0.16 ± 0.00[a,b]	0.22 ± 0.01[c]	0.18 ± 0.00[b]	0.29 ± 0.02[d]
C22:6n-3	0.23 ± 0.03[a,b]	0.17 ± 0.01[a]	0.43 ± 0.03[c]	0.35 ± 0.02[b,c]	0.99 ± 0.08[e]
Total C20 + C22 n-3 series	0.63 ± 0.05[a,b]	0.54 ± 0.02[a]	0.90 ± 0.05[c]	0.83 ± 0.02[b,c]	1.72 ± 0.13[e]
LN isomers	0.11 ± 0.01[b]	0.27 ± 0.02[e]	0.19 ± 0.02[c,d]	0.23 ± 0.02[d,e]	0.26 ± 0.01[e]
LA:ALA	12.85 ± 0.61[c]	9.89 ± 0.47[b]	17.85 ± 0.94[e,f]	7.62 ± 0.24[a]	9.94 ± 0.27[b]
AA:DHA	2.11 ± 0.11[d]	2.35 ± 0.10[e]	1.04 ± 0.04[b]	1.48 ± 0.05[c]	0.51 ± 0.03[a]
EPA:DHA	0.51 ± 0.03[d]	0.52 ± 0.03[d]	0.21 ± 0.01[a]	0.22 ± 0.01[a]	0.26 ± 0.01[a,b]

Country of origin	Mexico	Philippines	United Kingdom	United States
No. of women	46	54	44	49
C18:3n-3	1.05 ± 0.06[b,c]	0.43 ± 0.02[a]	1.22 ± 0.06[c,d]	1.05 ± 0.05[b,c]
C18:4n-3	0.04 ± 0.01[b,c]	0.03 ± 0.00[a,b,c]	0.03 ± 0.01[a,b,c]	0.01 ± 0.00[a,b]
C20:3n-3	0.04 ± 0.00[a,b]	0.04 ± 0.00[a]	0.05 ± 0.00[b]	0.04 ± 0.00[a]
C20:4n-3	0.07 ± 0.00[a]	0.09 ± 0.01[b,c]	0.11 ± 0.00[c]	0.08 ± 0.01[a,b]
C20:5n-3	0.07 ± 0.01[a]	0.15 ± 0.01[b]	0.11 ± 0.01[a]	0.07 ± 0.00[a]
C22:5n-3	0.16 ± 0.01[a,b]	0.23 ± 0.01[c]	0.18 ± 0.01[b]	0.14 ± 0.01[a]
C22:6n-3	0.26 ± 0.03[a,b]	0.74 ± 0.05[d]	0.24 ± 0.01[a,b]	0.17 ± 0.02[a]
Total C20 + C22 n-3 series	0.59 ± 0.05[a]	1.26 ± 0.08[d]	0.69 ± 0.02[a,b,c]	0.49 ± 0.03[a]
LN isomers	0.21 ± 0.01[c,d]	0.02 ± 0.01[a]	0.16 ± 0.01[c]	0.19 ± 0.01[c,d]
LA:ALA	16.91 ± 0.85[d,e]	19.51 ± 0.51[f]	8.95 ± 0.37[a,b]	15.44 ± 0.67[d]
AA:DHA	2.01 ± 0.10[d]	0.62 ± 0.03[a]	1.62 ± 0.07[c]	3.16 ± 0.13[f]
EPA:DHA	0.29 ± 0.01[b]	0.20 ± 0.01[a]	0.47 ± 0.02[d,c]	0.45 ± 0.02[c]

[a]Data are mean ± standard error. Means in the same row with different superscripts differ significantly at $P < 0.05$.

a high-maize diet, such as Mexico (19) and Chile (20). The Philippines presented an unusual profile, with low LA levels and high levels of lauric and myristic acids, most likely indicating consumption of diets limited in both essential FA and total fat. Our observations support a relationship between dietary LA intake and milk LA composition, although the direct dietary control of milk FA content cannot be proven in the present study. Studies using stable isotope-labeled LA demonstrated that approximately 30% of LA appearing in milk is derived from the isotopic label (21,22). These data suggest that both diet and a pool of LA that turns over relatively slowly[AUQ2] (for example, adipose tissue) are responsible for a substantial portion of LA appearing in milk.

In contrast to LA levels, which varied more than twofold

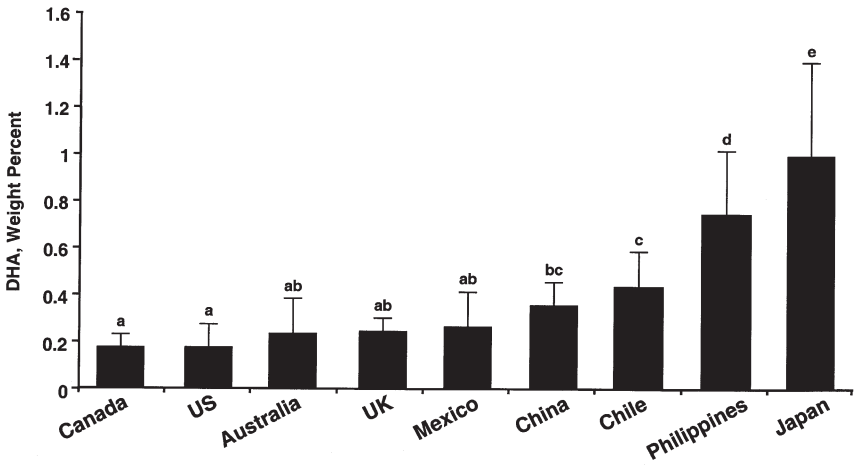

FIG. 2. DHA as weight percent of total FA in mature human milk of women from nine countries. Values are means ± SEM with 44 to 54 samples per group. Means with different superscripts are statistically different ($P < 0.05$).

TABLE 5
Human Milk FA Concentrations (mg/L)[a]

FA		LA		ALA		AA		DHA	
Country	n	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Australia	48	4233.1[a,b,c]	217.43	348.5[b]	18.28	148.7[b,c]	6.90	93.4[b]	14.41
Canada	48	4658.6[b,c]	250.15	501.7[c]	28.17	151.3[b,c]	8.68	73.3[b]	7.52
Chile	50	5073.7[b,c]	401.69	320.0[b]	28.73	116.4[a]	8.36	116.6[c]	8.25
China	50	6299.3[d]	369.74	863.5[d]	58.32	207.8[d]	12.05	149.8[d]	10.50
Japan	51	4546.8[b,c]	263.47	481.4[c]	36.76	141.9[b,c]	8.24	365.4e	39.78
Mexico	46	5415.5[c,d]	322.03	353.0[b]	28.01	140.7[b,c]	7.93	86.0[b]	10.17
Philippines	54	3524.8[a]	186.81	195.3[a]	15.45	171.5[c,d]	8.18	337.7[e]	35.06
United Kingdom	44	4034.1[a,b]	260.88	480.2[c]	35.92	141.5[b,c]	8.95	95.6[b,c]	9.12
United States	49	4421.2[a,b,c]	273.22	316.3[b]	25.47	132.9[a,b]	8.66	48.8[a]	5.16
Global mean	440	4683.9	102.70	427.1	13.88	150.7	3.13	156.2	8.68

[a]Data are mean and standard error. Means in the same column with different superscripts differ significantly at $P < 0.05$.

among countries, the mean AA level was relatively constant among all countries. The conversion of dietary, isotopically labeled LA to AA is very inefficient. Demmelmair *et al.* (22) found that only approximately 0.02% of an isotopically labeled oral LA dose appeared as milk AA over a 5-d period. The authors conclude that AA may be derived from sources other than dietary LA; however, concerns about a large endogenous pool of AA prevent definite conclusions from being drawn. The ratio of LA to AA in our human milk samples also argues against a direct, unregulated conversion of dietary LA to human milk AA. The LA:AA ratio varied almost twofold between countries (paralleling the proportion of LA in milk), but high proportions of milk LA did not predict high AA levels.

In the present study, elevated levels of milk DHA occurred in areas with high dietary consumption of fish. For example, countries with coastal collection sites and high fish or fish product consumption, such as Japan (23), the Philippines, and Chile (20), had DHA levels above 0.4%. In contrast, both Canada and the United States, where the interior collection sites had low fish consumption[AUQ3] (Edmonton, Alberta, and Tucson, Arizona, respectively), had very low DHA levels (below 0.2%). Dietary influences on milk DHA content are quite clear. For example, supplementation studies with fish oil (8) or DHA from a single cell oil[AUQ4] (9) rapidly elevated milk DHA levels in a dose-related manner. The AA:DHA ratio varied substantially in the present study, from 0.51:1 in Japan to 3.16:1 in the United States, as a result of relatively constant AA levels and highly variable DHA levels. Such variability in both DHA levels and in AA:DHA ratios means that there is no single, generally recognized standard for the composition of human milk LCP. Controlled randomized clinical studies with LCP-supplemented infant formulas have demonstrated that these FA may mediate improvements in both visual and cognitive function in preterm, and possibly also in term, infants (24–26). Positive results have been found when formulas contain 0.20–0.35 wt % DHA and an AA:DHA ratio between 1.5:1 and 2:1. The human milk LCP levels and AA:DHA ratios identified in the current study are clearly broader than the ranges evaluated in infant formula studies. To date there have not been dose-response studies to determine optimal DHA supplementation levels. Further research is necessary to evaluate the potential benefits of alternate addition levels.

The levels of lauric and myristic acids were similar among all countries, with the exception of the Philippines. The samples from the Philippines were obtained from women of low economic status; high milk levels of lauric and myristic acids suggest that the diet of these women was relatively high in carbohydrate and low in fat. Insull *et al.* (27) compared milk composition of women receiving either a fat-free diet or an isocaloric diet containing 40% of calories as fat. The lauric and myristic acid levels of milk in the group consuming the fat-free diet were much higher than levels in women consuming the fat-containing diet. Studies of milk composition from women whose typical diet was low in fat agree with our finding that lauric and myristic acid levels are elevated (19,28,29).

Oleic acid constituted the primary monounsaturated FA in all samples. Samples from Canada and China had the highest oleic acid content, confirming that these countries had diets characterized by relatively high consumption of canola or rapeseed oils (30,31). Diets high in oleic acid have previously been demonstrated to result in elevated human milk oleic acid levels (32). China also had milk erucic acid levels eight times higher than any other country and eicosenoic acid levels more than two times greater than any other country. These data are consistent with the consumption of rapeseed oil in China.

We have calculated the concentration of LA, ALA, AA, and DHA in the samples on a mg/L basis in order to compare levels found in human milk with regulatory requirements for infant formula. In order to estimate total FA concentrations, we made the assumption that the breast milk collection was representative of human milk during an entire 24-h period. A number of studies have evaluated diurnal variation of total human milk fat (reviewed in 33). In general, in Western women, milk fat tends to rise during the day with the peak occurring early in the evening. In developing countries, the peak tends to be early in the morning. Our collection time, during the afternoon, would be a midpoint in the diurnal cycle for all study populations. In terms of specific FA, Lammi-Keefe *et al.* (34) reported no significant diurnal variation in LA concentrations. The regulatory requirements for essential FA in formulas (generally expressed

as mg/100 kcal) have been converted to mg/L, using 670 kcal/L as a conversion factor, in order to permit direct comparisons with the present study's data. The LA content of all samples was greater than the U.S. FDA minimum requirement (35) of 300 mg/100 kcal (or 2,010 mg/L) and met current European Union (EU) guidelines (36) of providing from 300–1,200 mg/100 kcal (2,010–8,040 mg/L). Although the United States does not specify a requirement for ALA, the current EU Directive requires a minimum of 50 mg/100 kcal (335 mg/L). The overall mean ALA concentration in our survey would meet this level; however, the individual means of several countries including Chile, the Philippines, and the United States are below this minimum. The EU also requires the LA:ALA ratio to fall within the range of 5:1 to 15:1. Our data indicate that the mean of this ratio is greater than 15:1 in four of the countries surveyed (Chile, Mexico, the Philippines, and the United States). Smit *et al.* (37) conducted an extensive evaluation of human milk FA concentrations compared with regulatory standards and found that the levels of specific FA (LA, ALA, AA, DHA, lauric acid, and myristic acid) from the milk of numerous mothers fell outside of the regulatory guidelines.

In conclusion, we have determined the FA profiles of 440 human milk samples from nine countries. Levels of AA demonstrated only modest variation between countries. This relative consistency is of interest considering the large variation in habitual diets among those countries. In contrast, DHA demonstrated greater than a fivefold variation in breast milk composition among countries. The mean AA level in our survey of 0.41% is similar to the recommendation of the Child Health Foundation that at least 0.35% AA be added to formula (38). The Child Health Foundation also recommended at least 0.2% DHA be added to formula. The substantial variability of DHA among countries in our study, ranging from 0.17% to 0.99%, suggests that a formula addition level of 0.2% is an acceptable addition level, but future research is required to determine an optimal DHA level.

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Association of Ceramides in Human Plasma with Risk Factors of Atherosclerosis

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ABSTRACT: Atherosclerosis is a multifactorial disorder. Recent studies indicate that the plasma level of sphingomyelin, which yields ceramide, correlates with the risk of coronary heart disease. Therefore, ceramide, a well-known lipid causing apoptosis in various cell types, may contribute to atherogenesis. We examined the relationship between ceramide concentration and risk factors of atherosclerosis in normal human plasma using electrospray tandem mass spectrometry (LC-MS/MS). Major ceramides in human plasma were C24:0 and C24:1. The ceramide concentration showed a significant positive correlation with total cholesterol (TC) and triglycerides (TG). In addition, plasma ceramide level increased drastically at a high level of LDL cholesterol (more than 170 mg/dL). Our previous studies demonstrated that the sum of fragmented and conjugated apolipoprotein B-100 proteins (B-ox), which were products of a radical reaction of LDL as well as plasma, was a reliable index of atherosclerosis. B-ox showed a significant positive correlation with the plasma ceramide level. Based on these results, we propose that the ceramide level in human plasma is a risk factor at the early stages of atherosclerosis.

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Ceramide has been implicated in regulating cell cycle arrest, apoptosis, and cell senescence (1–3) and is reported to serve as an intracellular second messenger (4). One of the major sources of ceramide is from sphingolipid biosynthesis (1). *De novo* synthesis starting from serine palmitoyl-CoA transferase (SPT) also plays an important role in the biosynthesis of ceramide (5). A recent study indicated a significant positive correlation between plasma levels of sphingomyelin (SM) and the severity of coronary heart disease (6). Plasma SM levels increased in human familial hyperlipidemias (7). Recent studies demonstrated correlations between sphingomyelin and atherogenic risk factors of plasma in humans and inhibitions of SM and SPT reduced atherosclerotic lesion in apolipoprotein E knockout mice (8–10). These studies indicate that plasma SM levels and sphingomyelinase (SMase) activity may be risk factors for atherosclerosis, suggesting a role of ceramide in atherogenesis. However, a correlation between ceramide species in human plasma and atherosclerosis has never been examined.

Atherosclerosis is closely associated with modifications in LDL (11,12). Aggregation of LDL has been proposed to represent an essential and central process in atherogenesis (11), and LDL extracted from atherosclerotic lesions is aggregated or is prone to aggregate. Aggregated LDL in atherosclerotic lesions is enriched with ceramide (13), and LDL treated with SMase induces foam cell formation *in vitro* (13,14). Oxidative modification of LDL is also an important factor in the development of atherosclerosis (15). Uptake of oxidized LDL (oxLDL) by macrophages and smooth muscle cells leads to foam cell formation, which accumulates as lipid drops (16,17). Based on these reports, a correlation between ceramide and oxLDL is suggested.

Although LDL is composed of lipids, protein, and sugar chains, studies on the oxidation of LDL have focused mainly on lipid peroxidation (18). The protein part of LDL, apolipoprotein B-100 (apoB), is also reactive to radical oxidation and it undergoes fragmentation and conjugation (18–22). Among plasma proteins, apoB is unusually reactive to radical reactions compared to albumin and transferrin (21), and its reactivity is even comparable to α-tocopherol (21). In normal human serum, both fragmented and cross-linked apoB proteins are present and these oxidation reaction products of LDL tend to increase with age (23). In addition, we reported that B-ox, namely the sum of fragmented and conjugated apoB proteins determined by an immunoblot assay, showed a significant positive correlation with intima-media thickness of the carotid artery (IMT) and LDL cholesterol, and a negative correlation with HDL cholesterol and plasma vitamin C, which was an outstanding antioxidant in plasma (23,24). These reports suggest that B-ox is a reliable mechanism-based indicator of atherosclerosis. In this study, we examined for the first time correlations of ceramide with associated indices with atherosclerosis, including B-ox.

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Abbreviations: apoB, apolipoprotein B-100; IMT, intima-media thickness of the carotid artery; oxLDL, oxidized LDL; SM, sphingomyelin; SMase, sphingomyelinase; SPT, serine palmitoyl-CoA transferase; TC, total cholesterol; TG, triglyceride.

EXPERIMENTAL PROCEDURES

Materials. All solvents were purchased from Wako Pure Chemicals Co. (Osaka, Japan). All other reagents were obtained from Funakoshi Co. (Tokyo, Japan). Silica gel 60 TLC plates were purchased from Merck (Darmstadt, Germany). Vectastain ABC-PO (goat IgG) kit was purchased from Vector Lab. Inc. (Burlingame, CA, USA). Anti-human lipoprotein B goat IgG was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane filters were purchased from Millipore (Tokyo, Japan). Electrophoresis reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Subjects. We screened 100 Japanese inhabitants who participated in the annual local health-check program in Kisei Town (a rural area in Mie Prefecture, Japan). Table 1 gives the characteristics of the subjects. This study was approved by the Ethics Committee of International University of Health and Welfare and the study subjects gave informed consent to participate in the study.

Analytical method. Total cholesterol (TC), LDL cholesterol, HDL cholesterol, triglycerides (TG), phospholipid, and free fatty acid were measured using a commercially available diagnostic kit (Wako Pure Chemicals Co., Osaka, Japan).

Lipid extraction. The plasma (1 mL) was diluted with 1 mL of PBS, and 5 mL of a mixture of chloroform and methanol (2:1, vol/vol) was added. After vigorous shaking and centrifugation for 5 min at 3,400 *g*, the chloroform layer was collected. To the residue, 4 mL of chloroform was added and the extraction was performed again. The combined chloroform layer was evaporated with an evaporator and the resulting lipid was dissolved in 1 mL of chloroform. The recovery of exogenously added C16:0 ceramide to the plasma was satisfactorily in the range of 75–80% compared to 60–90% in a previous study (25).

Mass spectrometry. Quantitative measurement of ceramide species was made using a triple-quadrupole mass spectrometer (Finnigan MAT TSQ 7000). ESI-MS/MS was performed as previously described (25,26). HPLC was conducted with a μ-Bondasphere column (5 μC18 100A Waters). Elution was performed at a flow rate of 0.2 mL/min with a mixture of 5 mM ammonium formate, methanol, and tetrahydrofuran at a volume ratio of 1:2:7. The mobile phase stream was connected to the ionspray interface of an ESI-MS/MS system. Standards and cellular ceramide extracts were stored at –20°C. Mass analysis was performed in the positive mode in a heated capillary tube at 250°C with an electrospray potential of 4.5 kV, a sheath gas pressure of 70 psi, and a collision gas pressure of 1.6–2.0 mtorr. Under optimized conditions, monitoring ions were ceramide molecular species $(M+H)^+$ for the product ion at *m/z* 264 of the sphingoid base (25–28). Standards and samples were injected with 5 μL of 5 pmol/μL C8:0-ceramide as an internal standard for ESI-MS/MS. The quantity of each ceramide was calculated from each ceramide/C8:0-ceramide ratio, assuming that the calibration curve of ceramides bearing C16–24 acyl chains was similar to that of C16:0-ceramide. This assumption was verified previously (25). Each sample was analyzed in duplicate.

Electrophoresis, blotting, and immunoblot analysis. For electrophoresis, 15 μL of the sample was applied to SDS gel electrophoresis on a 4% polyacrylamide slab gel (1 mm thick) as described (22,29). Immunoblot analysis was performed as previously described previously (21–23). Proteins separated on the gel were eletrophoretically transferred to PVDF membrane filters and immunoblotting analyses of apoB were performed as previously described (23).

Statistical analysis. The data were expressed as mean ± SE. Results were analyzed by a Fisher test or using one-way ANOVA followed by Pearson's correlation test. Differences between group means were considered significant at $P < 0.05$.

RESULTS

Plasma ceramide concentration. Table 2 shows the distribution of ceramide species in human plasma. Major ceramides in plasma were C24:0 and 24:1. Other ceramides, C16:0, C18:0, C22:0, and C24:2, were also detected. These results were consistent with the report of Drobnik *et al.* (30), who reported that C24:0 was the main component, while C18:0 was minor, in the plasma.

Correlation of plasma ceramide level with clinical parameters of atherosclerosis. Total ceramide in the plasma showed a significant positive correlation with TC ($r = 0.651$, $P < 0.01$), TG ($r = 0.444$, $P < 0.01$), phospholipid ($r = 0.660$, $P < 0.01$), and free fatty acid ($r = 0.266$, $P < 0.01$) (Table 3). It did not correlate

TABLE 1
Characteristics of the Subjects[a]

Variable	
Age (years)	60.7 ± 1.2
Body mass index (kg/m^2)	23.5 ± 0.3
Total cholesterol (mg/dL)	219 ± 4
LDL cholesterol (mg/dL)	138 ± 3
HDL cholesterol (mg/dL)	61.5 ± 0.1
Triglyceride (mg/dL)	118 ± 6
Phospholipid (mg/dL)	241 ± 3
Free fatty acid (mg/dL)	0.65 ± 0.03
Blood glucose (mg/dL)	100 ± 4
Systolic blood pressure (mm Hg)	135 ± 2
Diastolic blood pressure (mm Hg)	79.2 ± 1.2

[a]Results shown are means ± SE, $n = 100$.

TABLE 2
Distribution of Ceramide Species in Human Plasma Samples[a]

Ceramide species	nmol/mL
C16:0	6.40 ± 0.21
C18:0	2.93 ± 0.17
C22:0	18.4 ± 0.8
C24:0	77.9 ± 3.0
C24:1	38.9 ± 1.2
C24:2	8.12 ± 0.35
Total ceramide	153 ± 5

[a]Results shown are means ± SE, $n = 100$.

TABLE 3
Correlation Coefficients of Plasma Ceramide Level with Other Parameters[a]

	Total ceramide	C24:0 ceramide
Total cholesterol	0.651[b] (100)	0.616[b] (100)
Triglyceride	0.444[b] (100)	0.390[b] (100)
Phospholipid	0.660[b] (100)	0.614[b] (100)
Free fatty acid	0.266[b] (100)	0.256[b] (100)
Systolic blood pressure	0.223[a] (100)	0.212[a] (100)
Diastolic blood pressure	0.157 (100)	0.159 (100)

[a]The numbers of subjects are shown in parentheses ([a]$P < 0.05$, [b]$P < 0.01$).

with HDL cholesterol. Total ceramide showed a significant positive correlation with systolic blood pressure ($r = 0.223$, $P < 0.05$), but not with diastolic blood pressure (Table 3). Moreover, positive correlations were observed between total ceramide and apoB, apoC, and apoE (data not shown). Ceramide of C24:0 showed a similar correlation (Table 3).

Relationship between LDL cholesterol and plasma ceramide. The subjects were divided into three groups according to LDL cholesterol level, because correlation of plasma ceramide level with TC was high and LDL cholesterol is the main carrier of cholesterol. The normal group had an LDL cholesterol level of less than 140 mg/dL ($n = 52$, LDL cholesterol average: 113.3 mg/dL), the moderate group had an LDL cholesterol level from 140 to 170 mg/dL ($n = 30$, LDL cholesterol average: 152.4 mg/dL), and the high group had an LDL cholesterol level of more than 170 mg/dL ($n = 20$, LDL cholesterol average: 184.4 mg/dL). The high group showed a significantly higher level of total ceramide than the normal and the moderate groups (Fig. 1).

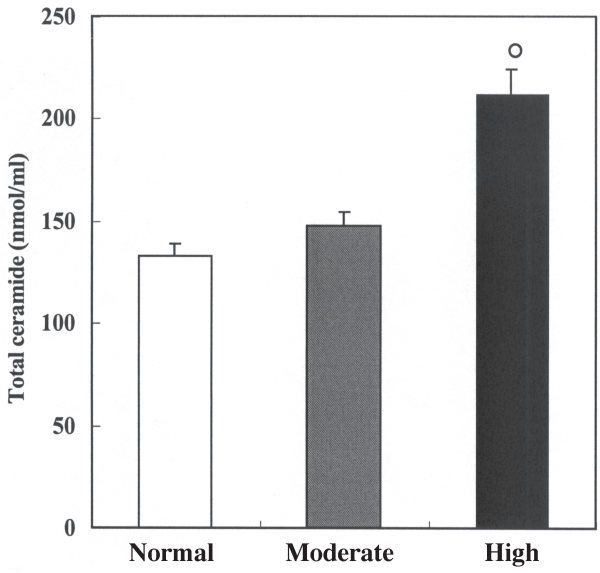

FIG. 1. The relationship between LDL cholesterol and total ceramide in plasma. The normal group had LDL cholesterol levels of less than 140 mg/dL. The moderate group had LDL cholesterol levels from 140 to 170 mg/dL. The high group had LDL cholesterol levels of more than 170 mg/dL. Values are expressed as means ± SE. ○P < 0.01 vs. normal group.

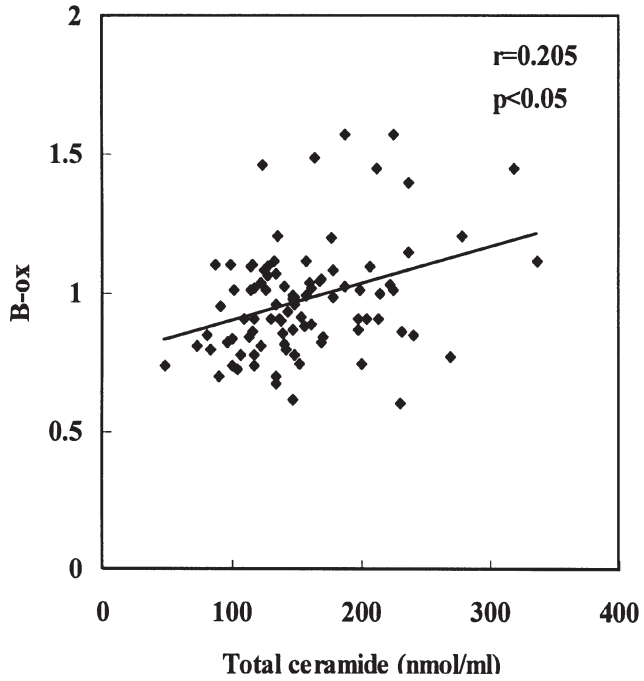

FIG. 2. Correlation coefficient of total ceramide with B-ox in plasma. We introduced an index "B-ox" to quantify the sum of fragmented and conjugated apolipoprotein B-100, products of radical reaction of LDL (23).

Correlation of plasma ceramide level with B-ox. Total ceramide showed a significant positive correlation with B-ox ($r = 0.205$, $P < 0.05$) (Fig. 2). Similar correlations ($r = 0.204$, $P < 0.05$) were observed when C24:0 was tested instead of total ceramide.

DISCUSSION

In this study, we measured the plasma levels of ceramides using ESI-MS/MS. Major ceramides of human plasma were C24:0 and C24:1. Plasma ceramide fatty acids of our study were different from that of Gorska *et al.* (31) and plasma SM fatty acids of Myher *et al.* (32,33). These differences may be due to the difference of analysis methods. Sphingolipids such as ceramide and sphingosine-1-phosphate are bioactive lipid mediators (34). The importance of sphingolipids as mediators in cardiovascular pathophysiology has recently been reported (35,36). In our results, ceramide showed significant positive correlations with TC and TG. These results suggested that an increase in plasma ceramide correlated directly with well-known lipid risk factors of atherosclerosis.

Since the correlation coefficient of cholesterol and total ceramide was high, we examined the relationship between LDL cholesterol and total ceramide in plasma. The subjects were divided into three groups according to LDL cholesterol level. As a result, the high group in LDL cholesterol showed dominantly high ceramide level. Ceramide can be generated by hydrolysis of sphingomyelin, catalyzed by SMase (1). It was reported that treatment of LDL with SMase induces aggregation and foam

cell formation (13,14,37). Therefore, there might be correlation between ceramide increases in plasma and high level of aggregated LDL. Thus, a ceramide increase in plasma may contribute to atherogenesis.

OxLDL is assumed to play a key role in the inflammatory process in atherosclerotic lesions (15). The protein part of LDL, apoB, undergoes fragmentation and cross-linking by radical oxidation (19,20,22,38–41). ApoB has an unusually high reactivity to radical reactions among plasma proteins and its reactivity is almost comparable to α-tocopherol (21), a typical antioxidant. The extremely high reactivity of apoB to radical reactions explains why fragmented and cross-linked apoB proteins exist in normal human plasma (22,23). These studies indicate that B-ox representing the fragmentation and cross-linkage pattern of apoB reflects the oxidative stress in individuals, and that it may be a mechanism-based index for atherosclerosis (23). Ceramide concentrations in plasma showed a significantly positive correlation with B-ox. It is possible that oxidation of LDL stimulates ceramide production and *vice versa*.

OxLDL has been shown to promote macrophage recruitment and apoptosis of endothelial cells, as well as to activate the SMase-ceramide pathway in vascular smooth muscle cells (42–44). Therefore, it is suggested that ceramide, which is increased in oxLDL, induces apoptosis in endothelial cells and is related to atherogenesis. However, oxLDL-induced activation of the SMase-ceramide pathway has not yet been fully studied (45). The relationship between ceramide and oxLDL, especially B-ox, should be further examined.

The results of our study show for the first time that ceramide concentrations in human plasma had a significantly positive correlation with products of a radical reaction of LDL, and lipid markers that associated with atherogenesis. Ceramides have been shown to cause apoptosis in a variety of cells and apoptosis of endothelial cells is widely implicated in the early stage of atherosclerosis. Therefore, an increase in plasma ceramide may be a risk factor at the early stages of atherosclerosis. It is necessary to examine the underlying mechanism by biochemical studies.

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Regiospecificity Profiles of Storage and Membrane Lipids from the Gill and Muscle Tissue of Atlantic Salmon (*Salmo salar* L.) Grown at Elevated Temperature

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ABSTRACT: Regiospecific and traditional analysis, of both storage and membrane lipids, was performed on gill, white muscle, and red muscle samples taken from Atlantic salmon (*Salmo salar*) to gauge the effect of elevated water temperature. The fish, fed a commercial diet, were held at an elevated water temperature of 19°C. Total n-3 PUFA, total PUFA, and n-3/n-6 and unsaturated/saturated fatty acid (UFA/SFA) ratios in the FA profile of the total lipid extract in the white muscle were fairly low compared with fish grown at 15°C. Adaptation of structural and storage lipids at elevated temperatures was shown by a significant ($P < 0.01$) reduction in PUFA especially in the percentage of EPA (6–8%). Further adaptation was indicated by the percentages of SFA, which were significantly ($P < 0.05$) higher in gill (56%) and white muscle (58%) polar lipid fractions and coincided with lower percentages of n-3, n-6, and total PUFA. The regiospecific profiles indicated a high affinity of DHA to the *sn*-2 position in both the TAG (61–68%) and polar lipid (35–60%) fractions. The combination of detailed regiospecific and lipid analyses demonstrated adaptation of cell membrane structure in Atlantic salmon grown at an elevated water temperature.

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Examination of the regiospecificity profile of the lipid classes of Atlantic salmon (*Salmo salar* L.) at an elevated temperature of 19°C has yet to be fully investigated. Regiospecificity is the position of individual FA on the glycerol backbone of both storage and membrane lipids. These lipids are present and act in the body as complex molecules and therefore should be studied as whole molecules. Traditional lipid class and FA profiling provides important compositional information; however, it does not reveal the regiospecific details of the molecule, which can play a key role in their function including bioactivity in humans and other animals (1–3).

The FA composition of lipids is important, but so too are the particular combinations of FA within individual phospholipid classes (4). Up to 70 different polar lipid (PL) molecular species have been identified for a range of different fish species (5–8), and their diversity and abundances can depend on species, tissue sampled, dietary and environmental factors (9). The composition of these molecular species and their role in the cell membrane can be influenced by many factors, but little is known about how they function in fish.

With advances in analytical and computing facilities, new techniques and methods can be used to examine intact lipids and focus on the regiospecific distribution of specific FA. Electrospray ionization reversed-phase liquid chromatography-MS (ESI RP-LCMS) provides a rapid and accurate method to measure molecular species. This method provides regiospecific information on how membranes adapt to different factors such as temperature, hormonal states, and photoperiod. This method has had only limited application to Atlantic salmon (10) and it has yet to be used to explore a range of tissues.

The regiospecific distribution of the TAG fraction of salmon has been examined by ^{13}C NMR (11). The regiospecific distribution of the TAG fraction may affect its physical characteristics and potentially have a profound influence on function and resultant bioactivity to consumers (1,2). It was first shown in infant nutrition that the regiospecific distribution of FA in the diet affects their absorption (3). Therefore, absorption of the long-chain PUFA (LC-PUFA) by human consumers of salmon may be affected by position on the TAG molecule.

Regiospecific analysis has been performed for other fish species *via* destructive and/or time-consuming methods, such as using lipases (12,13) and TLC combined with HPLC (5,7,8,14). These two techniques allow a rapid and detailed examination of intact membrane and storage lipids. In studying intact molecules it is possible to determine the regiospecificity of the FA and whether the molecular structure plays a role in their function, storage, and absorption, in particular in regard to n-3 LC-PUFA.

The physical properties of cell membranes and their lipid content can be affected by temperature (13,15–18). Fish may exploit the structural diversity of lipids within their membranes to adapt to physical properties, such as change in ambient temperature. The tailoring of membrane lipid composition is probably the most ubiquitous and continuously graded cellular response to temperature change (13). Most studies have investigated Atlantic salmon lipids within a temperature range of

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Abbreviations: $CDCl_3$, deuterated chloroform; DPA, docosapentaenoic acid; ESI-RP-LCMS, electrospray ionization reversed-phase liquid chromatography-MS; LC, long-chain; MUFA, monounsaturated FA; PL, polar lipid; PLFA, polar lipid FA; SFA, saturated FA; TLE, total lipid extract; tr, trace amounts; UPL undetermined PL; UFA, unsaturated FA.

2–12°C (19–22), but none have measured salmon at higher temperatures such as 19°C, a temperature that is now commonly encountered by farmed salmon in Tasmanian waters and that approaches their upper threshold for survival (23).

This is the first detailed examination, including regiospecific profiling, of the membrane and storage lipids of salmon from a feeding experiment performed at an elevated temperature of 19°C. This study aims to combine and apply these two regiospecific analyses for both storage and structural lipids from a range of different tissues. With global waters increasing in temperature, this study will indicate possible adaptive changes in salmon lipid makeup at elevated temperature.

EXPERIMENTAL PROCEDURES

Experimental system. The experiment was conducted at the School of Aquaculture, University of Tasmania. Atlantic salmon parr were obtained from Wayatinah Salmon hatchery (SALTAS, Tasmania, Australia), and 14 fish per tank were randomly stocked into three 300-L tanks in a partial recirculation system (24). The tanks were held at a constant temperature of 19°C in 30 ppt seawater (24) with a photoperiod of 16:8, light/dark. The water was treated through physical, biological, and UV filters. Dissolved oxygen, pH, ammonia, nitrate, and nitrite were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon (25). A commercially extruded feed (Skretting, Tasmania, Australia) was fed to excess twice a day (0900–1000 and 1600–1700 h) by automatic belt feeders. The salmon had an initial weight of 139.6 + 1.5 g (mean ± SE; $n = 6$), were grown for 105 d, and reached an average final weight of 510.2 ± 13.2 g. Salmon weighing 420.2 ± 6.3 g, $n = 3$, maintained at 15°C and with a similar history and fed on the same diet, provided a reference group that represented baseline temperature conditions.

Sampling. On the last day (day 104) of the trial, fish were killed by a blow to the head after immersion in anaesthetic (benzocaine, 100 mg/L) and two fish were sampled from each tank. Samples of gill tissue were dissected from the first gill arch. Red (average sample weight 1.3 ± 0.2 g, $n = 6$) and white muscle (average sample weight 2.4 ± 0.1 g, $n = 6$) samples were taken from below the dorsal fin. Samples were immediately frozen and stored at –80°C until analysis. Salmon at 15°C were similarly sampled. The experiment was conducted in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0007719).

Lipid extraction, fractionation, and FA analysis. Samples were freeze-dried and extracted by a modified Bligh and Dyer protocol (26). A single-phase extraction, $CHCl_3$/MeOH/H_2O (1:1:0.9, by vol), was used to yield a total lipid extract (TLE).

Lipid classes were analyzed by an Iatroscan MK TLC-FID analyzer (Iatron Laboratories, Tokyo, Japan). Samples were spotted onto silica gel SIII Chromarods (5 mm particle size) and developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane/diethyl ether/acetic acid (60:17:0.1, by vol) (27). After development for 25 min, the Chromarods were oven-dried and analyzed immediately to minimize adsorption of atmospheric contaminants. Lipid classes were quantified by DAPA software (Kalamunda, Western Australia, Australia). The FID was calibrated for each compound class: PC; cholesterol; cholesteryl ester; oleic acid; hydrocarbon (squalene); wax ester (derived from fish oil); TAG (derived from fish oil); and DAG ether (purified from shark liver oil). To analyze the PL fraction, a second solvent system, chloroform/methanol/water (80:35:3 by vol), was used (28). The Iatroscan was calibrated with PC with equivalent response factors assumed across the PL classes. Retention factors were measured for each phospholipid and compared with standards (Sigma-Aldrich).

An aliquot of TLE was transmethylated in methanol/chloroform/hydrochloric acid (10:1:1, by vol) for 1 h at 100°C. After addition of water, the mixture was extracted three times with hexane/chloroform (4:1, vol/vol) to obtain FAME. FAME were concentrated under nitrogen and treated with N,O-bis(trimethylsilyl)-trifluoroacetamide (50 mL, 60°C, 1 h) to convert hydroxyl groups to their corresponding trimethyl silyl ethers. Samples were made up to a known volume with an internal injection standard (23:0 or 19:0 FAME) and analyzed by GC using an Agilent Technologies 6890N gas chromatograph (Palo Alto, CA) equipped with an HP-5 cross-linked methyl silicone fused-silica capillary column (50 m × 0.32 mm i.d.) and an FID. Helium was used as the carrier gas. Samples were injected, by a split/splitless injector and an Agilent Technologies 7683 Series auto sampler in splitless mode, at an oven temperature of 50°C. After 1 min the oven temperature was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C min^{-1} and finally to 300°C at 5°C min^{-1}. Peaks were quantified by Agilent Technologies GC ChemStation software. Individual components were identified by mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC results are typically subject to an error of ±5% of individual component area. GC-MS analyses were performed on a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector with Thermoquest Xcalibur software (Austin, TX). The GC was fitted with a capillary column similar to that described above.

Fractionation of lipid classes was accomplished by silicic acid column chromatography. The TLE was applied to a 1 g column of silicic acid (preheated to 100°C, 1 h) and separated into neutral lipids, glycolipids, and PL in a stepwise elution of chloroform (10 mL), acetone (20 mL), and methanol (10 mL), respectively (29).

Membrane lipid analysis: ESI-RP-LCMS. PL species were analyzed by LCMS by negative ion ESI. A Waters Alliance 2690 high-performance liquid chromatograph fitted with a Nova-Pak RP C18 column (3.9 × 150 mm) was coupled to a Finnigan LCQ ion trap mass spectrometer fitted with an electrospray source. The mobile phase gradient was methanol/0.25 M ammonium acetate (pH 5), (96:4 vol/vol) to methanol/0.25 M ammonium acetate (pH 5)/hexane (86:4:10 by vol) at 20 min, then to methanol/hexane (80:20 vol/vol) at 25 min, which was held for 10 min. The flow rate was 1 mL min^{-1}. Alternating normal scans in the range *m/z* 600–950 and data-dependent

MS/MS product ion scans were acquired. The MS/MS scans isolated the strongest ion observed, and a collision energy of 40% was applied with all product ions monitored. The capillary temperature was 275°C, sheath gas pressure 600 kPa, auxiliary gas pressure 275 kPa, and the needle voltage was 4 kV. Minor molecular species (>0.1 across all tissues) are not included to simplify the analysis. PL species and regiospecificity were identified by MS and MS/MS breakdown products according to published data (10).

Storage lipid analysis: ^{13}C NMR spectroscopy. Acyl group positional distributions on TAG were determined quantitatively by ^{13}C NMR on a 400 MHz Varian (Palo Alto, CA) Inova Wide Bore spectrometer, with either a Broadband 10 mm probe or a 3 mm MAS Nanoprobe at 25.0 ± 0.1°C. A π pulse at 100.516 MHz, 128 K acquisition with 2.5 s relaxation delay and full nuclear Overhauser effect enhancement and decoupling sequence was employed. Samples were dissolved in 99.96% deuterated chloroform ($CDCl_3$) with *ca.* 0.025 M $Cr(acac)_3$ [tris(acetylacetonato)chromium(III)] as a relaxation agent and brought to a volume of 0.6 mL. TAG fractions that were <10 mg were dissolved in 20–35 μL of $CDCl_3$/$Cr(acac)_3$ in a glass rotor and filled to a total volume of 40 μL and spun at 3.000 ± 0.002 kHz. The number of transients collected was 512 to 8192. A 0.12 Hz Gaussian and a 0.15 Hz Lorentzian window function was applied to the free induction decay prior to transformation, and polynomial baseline correction and drift correction were applied to each spectrum. Peak chemical shifts were referenced to $CDCl_3$ at 77.16 ppm (30).

Statistical analysis. Mean values were reported plus or minus the SEM. Percentage data were arcsin transformed prior to analysis. Normality and homogeneity of variance were confirmed, and a comparison between means was achieved by one-way ANOVA. Multiple comparisons were achieved by Tukey-Kramer HSD (honestly significant difference). Significance was accepted as probabilities of 0.05 or less. Statistical analysis was performed using SPSS (statistical package for social science) for windows version 11 (SPSS, Chicago, IL).

RESULTS

Lipid class. TAG accounted for the largest proportion of the lipid in the diet (88.2%) and in the tissues (81.9–94.1%) (Table 1). The red muscle had significantly ($P < 0.05$) higher percentage of TAG compared with the gill and white muscle, but diet was not significantly different from any sampled tissue. The PL value varied across the tissues and was significantly ($P < 0.05$) higher in the gills (15.8%) and the white muscle (15.5%) than in red muscle (4.8%). The diet contained 8.4% PL and was not significantly different from any tissues. FFA were significantly ($P < 0.01$) higher in the diet (1.7%).

FA profiles of total lipid. The major (>10%) dietary FA were 16:0, 18:1n-9, EPA, and DHA, with 14:0 and 16:1n-7 present at moderate percentage abundances (5–10%) (Table 1). Of all the FA, 16:0, 18:1n-9, and DHA were at high proportions across all tissues, whereas 14:0, 16:1n-7, and EPA were present at moderate percentages. In addition, 18:0 was present at moderate abundance in white muscle and gills. The TLE FA profile of the three tissues reflected closely the profiles of the diet. This was judged by the small statistical difference between the diet and the tissues. There were no significant differences in the relative percentage of monounsaturated FA (MUFA) in any tissue or the diet. The major exception was EPA, which showed a significantly ($P < 0.01$) lower percentage in all the salmon tissues compared with the diet.

A significantly ($P < 0.05$) lower unsaturated FA/saturated FA (UFA/SFA) ratio in the white muscle and significantly ($P < 0.05$) lower n-3/n-6 ratios were observed in all tissues compared with the diet. There was a significant percentage reduction in total n-3 PUFA ($P < 0.01$), total n-6 PUFA ($P < 0.05$), and total PUFA ($P < 0.01$) in white muscle compared with the diet and red muscle.

15°C fish. Lipid class and FA profiles for fish grown at a water temperature of 15°C are shown in Table 2. These fish had a very similar history, and results are included to provide comparison with the fish grown at the elevated temperature of 19°C. The lipid class profiles for the 15°C fish are very similar to 19°C fish (Table 1). The FA profiles showed that in the red and white muscle there was decreased SFA and increased n-3 PUFA and total PUFA in the 15°C fish (Fig. 1).

Lipid class composition of PL. The major (>10%) phospholipid classes in all samples were PE and PC (Table 3). Both PE and PS were significantly ($P < 0.05$) lower in all tissues compared with the diet. PI was significantly ($P < 0.05$) higher in red muscle. Unidentified PL (UPL) consisted of several very polar peaks, which may have included sphingomyelin and lysophosphatidyl derivatives. UPL was significantly higher in all salmon tissues ($P < 0.05$) compared with the diet.

FA profiles of PL. The major (>10%) dietary polar lipid FA (PLFA) were 16:0, 18:1n-9, 20:5n-3, and 22:6n-3, with 14:0, 16:1n-7, and 18:2n-6 present at moderate percentages (5–10%) (Table 3). The major PLFA for the gills were 16:0, 18:0, and 18:1n-9, with 14:0, 16:1n-7, and 22:6n-3 also present in moderate abundance. The major PLFA for white muscle were 16:0, 18:0, 18:1n-9, and 22:6n-3 with 14:0, and 20:5n-3 also present in moderate abundance. The major FA for red muscle PL were 16:0, 18:1n-9, and 22:6n-3, with 14:0, 18:0, and 20:5n-3 also present in moderate abundance.

Accumulation of PUFA was not observed in the PL fraction. This was shown by the decrease in total PUFA percentage across all tissues compared with the FA profile of the 19°C fish. Over 50% of the PLFA in the gill and white muscle were saturates (especially 16:0: 34.4 and 37.1%, respectively), which were significantly ($P < 0.01$) higher than in the red muscle.

Gill PLFA showed a statistically higher percentage of MUFA, especially 18:1n-9 (17.7%), 18:1n-7 (3.9%), and total MUFA (30.2%). This coincided with significantly ($P < 0.01$) lower relative abundances of n-3, n-6, and total PUFA. Lower percentages of n-3, n-6, and total PUFA occurred in the white muscle compared with red muscle.

A significantly ($P < 0.01$) lower percentage of DHA was present in the gills (5.2%) than white muscle (10.5%), but red muscle was significantly ($P < 0.05$) higher (20.1%). The

TABLE 1
FA and Lipid Class Composition and Content of the Total Lipid Fraction of a Commercial Formulated Diet and White and Red Muscle and Gill Tissue of Atlantic Salmon (*Salmo salar*) Fed That Diet When Held at 19°C

	Percent composition			
Sample	Diet	Gill	White muscle	Red muscle
SFA				
14:0	6.4 ± 0.1[b]	5.2 ± 0.1[a]	6.3 ± 0.3[b]	6.5 ± 0.2[b]
16:0	19.4 ± 0.3[a]	19.0 ± 0.1[a]	20.3 ± 0.4[b]	19.3 ± 0.4[a,b]
18:0	4.2 ± 0.1[a]	5.4 ± 0.1[b]	5.0 ± 0.0[a,b]	4.6 ± 0.1[a,b]
Other SFA[d]	1.9 ± 0.0	1.9 ± 0.0	2.4 ± 0.0	2.3 ± 0.0
Total SFA	32.0 ± 0.4	31.5 ± 0.1	34.0 ± 0.7	32.6 ± 0.6
MUFA				
16:1n-7*c*	7.0 ± 0.1	6.7 ± 0.1	7.3 ± 0.4	8.0 ± 0.3
18:1n-9*c*[e]	14.1 ± 0.3	15.7 ± 0.2	16.7 ± 0.5	16.7 ± 0.3
18:1n-7*c*	3.0 ± 0.0	3.5 ± 0.0	3.6 ± 0.2	3.6 ± 0.1
20:1n-9*c*	1.6 ± 0.1	1.6 ± 0.0	1.8 ± 0.1	1.8 ± 0.0
Other MUFA[f]	3.6 ± 0.0	3.9 ± 0.0	3.2 ± 0.0	2.9 ± 0.0
Total MUFA	29.3 ± 0.1	31.3 ± 0.0	32.6 ± 0.2	33.1 ± 0.1
n-3 PUFA				
18:4n-3	2.8 ± 0.1[b]	2.2 ± 0.0[b]	0.4 ± 0.4[a]	2.4 ± 0.0[b]
20:4n-3	0.9 ± 0.0	1.2 ± 0.0	1.0 ± 0.0	1.2 ± 0.0
20:5n-3 EPA	12.7 ± 0.3[b]	8.0 ± 0.2[a]	6.7 ± 0.6[a]	7.3 ± 0.3[a]
22:6n-3 DHA	11.0 ± 0.9[a,b]	13.1 ± 0.3[b]	10.4 ± 1.2[a,b]	11.0 ± 0.6[a]
22:5n-3 DPA	2.0 ± 0.0	3.2 ± 0.1	2.7 ± 0.2	3.0 ± 0.1
Other n-3 PUFA[g]	0.6 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0
Total n-3 PUFA	30.0 ± 0.2[b]	28.6 ± 0.1[b]	21.9 ± 0.1[a]	25.6 ± 0.2[b]
n-6 PUFA				
18:2n-6	4.2 ± 0.1	4.0 ± 0.0	4.1 ± 0.2	4.4 ± 0.1
20:4n-6	0.8 ± 0.0	1.2 ± 0.0	0.7 ± 0.1	0.7 ± 0.0
22:5n-6	0.4 ± 0.0[b]	0.4 ± 0.0[b]	0.3 ± 0.0[a]	0.3 ± 0.0[a]
Other n-6 PUFA[h]	0.6 ± 0.0	1.2 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
Total n-6 PUFA	6.0 ± 0.0[a]	6.9 ± 0.0[b]	6.0 ± 0.1[a]	6.3 ± 0.0[a,b]
Other PUFA[i]	1.8 ± 0.0	1.8 ± 0.0	3.8 ± 0.5	2.4 ± 0.0
Total PUFA	37.8 ± 0.2[b]	37.2 ± 0.3[b]	31.7 ± 0.2[a]	34.3 ± 0.1[b]
n-3/n-6	5.0 ± 0.1[c]	4.2 ± 0.1[b]	3.7 ± 0.1[a]	4.1 ± 0.2[a,b]
UFA/SFA	2.1 ± 0.0[b]	2.2 ± 0.0[b]	1.9 ± 0.0[a]	2.1 ± 0.0[b]
Lipid class				
TAG	88.2 ± 0.4[a,b]	81.8 ± 1.0[a]	83.8 ± 4.9[a]	94.1 ± 1.0[b]
FFA	1.7 ± 0.1[c]	0.6 ± 0.0[b]	0.2 ± 0.1[a]	0.6 ± 0.1[b]
ST	1.3 ± 0.1[b]	1.4 ± 0.1[b]	0.5 ± 0.1[a]	0.5 ± 0.1[a]
PL	8.4 ± 0.5[a,b]	15.8 ± 1.0[b]	15.5 ± 4.9[b]	4.8 ± 0.9[a]
Total lipid (g/kg WW)[j]	262.1 ± 3.8	69.4 ± 3.2	58.2 ± 5.8	220.0 ± 12.2

[a,b,c]Mean values across the row not sharing a common superscript were significantly different ($P < 0.05$) as determined by Tukey–Kramer HSD (honestly significant difference); $n = 6$.
[d] "Other SFA" includes 15:0, 17:0, 20:0, 22:0, and 24:0.
[e] Includes 18:3n-3 (minor component)
[f] "Other MUFA" includes 16:1n-9, 16:1n-5, 18:1n-5, 20:1n-7, 22:1n-9, 22:1n-11, and 24:1n-9.
[g] "Other n-3 PUFA" include 21:5n-3 and 24:6n-3.
[h] "Other n-6 PUFA" include 20:2n-6, 20:3n-6, 22:4n-6, and 24:5n-6.
[i] "Other PUFA" include 16:2n-4, 16:3n-4, and 18:2n-9.
[j] Determined by TLC-FID. SFA, saturated FA; MUFA, monosaturated FA; DPA, docosapentaenoic acid; ST, sterol; PL, polar lipid; WW, wet weight; UFA, unsaturated FA; *c*, *cis*.

UFA/SFA ratio was significantly ($P < 0.05$) lower in the gill and white muscle fractions. The n-3/n-6 ratio was significantly ($P < 0.05$) lower in the gills compared with the red and white muscle ($P < 0.05$).

Regiospecific analysis of the membrane lipids. The major PL (>10%) species for the gills were PC 16:0/18:1 (denotes 16:0 at *sn*-1 and 18:1 at *sn*-2 on the PC molecule), PE 18:0/18:1, and PE 18:0/22:6, with moderate percentages (5–10%) of PC 16:0/22:6 and PE 16:0/22:6 (Table 4). The gills had the highest proportion of SFA/MUFA, MUFA/MUFA, and MUFA/PUFA profiles (Fig. 1). The major PL species for white muscle was PE 18:0/22:6, with moderate percentages of PC 16:0/18:1, PC 16:0/22:6, and PE 18:0/20:5. The major PL species for red muscle were PC 16:0/22:6, PE 16:0/22:6, and

TABLE 2
FA and Lipid Class Composition and Content[a] of the Total Lipid Fraction of White and Red Muscle and Gill Tissue of Atlantic Salmon (*Salmo salar)* Fed Commercial Formulated Diet When Held at 15°C

	Percent composition		
	Gill 15°C	White 15°C	Red 15°C
SFA			
14:0	4.8 ± 0.5	5.9 ± 0.1	5.8 ± 0.2
16:0	21.3 ± 0.4	17.3 ± 0.1	18.7 ± 0.5
18:0	5.9 ± 0.4	4.1 ± 0.1	2.8 ± 1.4
Other SFA[b]	2.1 ± 0.2	2.1 ± 0.2	2.0 ± 0.1
Total SFA	34.1 ± 1.0	29.4 ± 0.3	29.3 ± 0.6
MUFA			
16:1n-7*c*	7.4 ± 0.5	8.5 ± 0.1	8.1 ± 0.1
18:1n-9*c*[c]	15.9 ± 0.4	15.6 ± 0.4	14.7 ± 0.4
18:1n-7*c*	4.2 ± 0.1	3.7 ± 0.0	3.7 ± 0.0
20:1n-9*c*	1.6 ± 0.1	1.7 ± 0.1	1.4 ± 0.1
Other MUFA[d]	3.2 ± 0.1	3.3 ± 0.1	3.2 ± 0.1
Total MUFA	32.2 ± 1.1	32.9 ± 0.6	31.1 ± 0.5
n-3 PUFA			
18:4n-3	1.9 ± 0.2	2.5 ± 0.0	2.4 ± 0.1
20:4n-3	1.1 ± 0.1	1.3 ± 0.0	0.9 ± 0.4
20:5n-3 EPA	7.1 ± 0.1	7.1 ± 0.3	7.9 ± 0.5
22:6n-3 DHA	14.1 ± 1.5	13.9 ± 0.3	16.2 ± 0.6
22:5n-3 DPA	2.2 ± 0.2	3.4 ± 0.1	3.2 ± 0.1
Other n-3 PUFA[e]	0.8 ± 0.1	1.3 ± 0.1	1.1 ± 0.1
Total n-3 PUFA	27.2 ± 1.2	29.4 ± 0.7	31.8 ± 0.8
n-6 PUFA			
18:2n-6	4.3 ± 0.3	5.2 ± 0.1	4.9 ± 0.0
20:4n-6	2.2 ± 0.5	0.8 ± 0.0	0.9 ± 0.1
22:5n-6	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Other n-6 PUFA[f]	0.9 ± 0.1	1.5 ± 0.1	1.1 ± 0.1
Total n-6 PUFA	7.7 ± 0.2	7.8 ± 0.1	7.3 ± 0.0
Other PUFA[g]	0.5 ± 0.1	1.0 ± 0.1	0.9 ± 0.2
Total PUFA	35.4 ± 1.2	38.2 ± 0.7	40.1 ± 0.7
n-3/n- 6	3.5 ± 0.2	3.8 ± 0.1	4.3 ± 0.1
UFA/SFA	2.0 ± 0.1	2.4 ± 0.0	2.4 ± 0.1
Lipid class			
TAG	79.2 ± 0.3	84.2 ± 2.1	96.1 ± 2.1
FFA	0.7 ± 0.0	0.2 ± 0.1	0.4 ± 0.1
ST	1.6 ± 0.1	0.6 ± 0.2	0.3 ± 0.1
PL	18.5 ± 0.2	15.0 ± 0.8	3.2 ± 0.7
Total lipid (g/kg WW)[h]	31.5 ± 8.4	57.9 ± 3.2	222.7 ± 6.7

[a]Mean values across the row not sharing a common superscript were significantly different ($P < 0.05$) as determined by Tukey–Kramer HSD; $n = 6$. For abbreviations see Table 1.
[b]Other SFA includes 15:0, 17:0, 20:0, 22:0, and 24:0.
[c]Includes 18:3n-3 (minor component).
[d]Other MUFA includes 16:1n-9, 16:1n-5, 18:1n-5, 20:1n-7, 22:1n-9, 22:1n-11, and 24:1n-9.
[e]Other n-3 PUFA include 21:5n-3 and 24:6n-3.
[f]Other n-6 PUFA include 20:2n-6, 20:3n-6, 22:4n-6, and 24:5n-6.
[g]Other PUFA include 16:2n-4, 16:3n-4, and 18:2n-9.
[h]Determined by TLC-FID.

PE 18:0/22:6, with moderate percentages of PE 18:0/20:5 and PE 18:1/22:6.

Faint signals of phosphatidylglycerol (PG) species were detected by LCMS, in particular PG 16:0/18:2, PG 16:0/18:1, PG 18:0/18:2, and PG 18:0/18:1, in some samples. Small amounts of PG 16:0/18:1 were found in all samples, as was sphingomyelin.

Regiospecific analysis of storage lipid: ^{13}C NMR. Where random distribution of a FA occurs across the TAG molecule, 33% of any FA would be expected at the *sn*-2 position. Since the TAG molecule has a symmetric plane, the relative proportions at the *sn*-1 and *sn*-3 positions cannot be identified by ^{13}C NMR. DHA had a high affinity with the *sn*-2 position (62.1–68.3% across the tissues). Docosapentaenoic acid (DPA) (37.3–39.6%) and 18:4n-3 (42.1–46.3%) also had an affinity to the *sn*-2 position. EPA (34.2–36.0% *sn*-2) and 20:4 (32.6–35.3% *sn*-2) had even distribution across the molecule and did not show a preferred regiospecific position (Table 5).

TABLE 3
FA and Lipid Class Composition and Content of the Polar Lipid Fraction of White and Red Muscle and Gill Tissue of Atlantic Salmon *Salmo salar* Fed Commercial Formulated Diet When Held at 19°C

	Percent composition		
Sample	Gill	White muscle	Red muscle
SFA			
14:0	5.6 ± 0.3	5.2 ± 0.3	5.0 ± 0.4
16:0	34.4 ± 0.5[b]	37.1 ± 5.8[b]	23.2 ± 0.5[a]
18:0	12.8 ± 1.8	14.1 ± 5.6	7.4 ± 1.1
Other SFA[d]	3.0 ± 0.1	2.3 ± 0.1	1.9 ± 0.0
Total SFA	55.8 ± 0.7[b]	58.6 ± 3.0[b]	37.6 ± 0.5[a]
MUFA			
16:1n-7*c*	5.3 ± 0.2[b]	3.8 ± 0.1[a]	4.7 ± 0.5[a,b]
18:1n-9*c*[e]	17.7 ± 0.3[b]	12.1 ± 1.2[a]	12.3 ± 0.8[a]
18:1n-7*c*	3.9 ± 0.1[b]	3.0 ± 0.5[a,b]	2.7 ± 0.3[a]
20:1n-9*c*	0.9 ± 0.1[b]	0.2 ± 0.3[a]	0.9 ± 0.1[b]
Other MUFA[f]	2.6 ± 0.0	1.0 ± 0.1	3.0 ± 0.0
Total MUFA	30.2 ± 0.1[c]	20.2 ± 0.4[a]	23.7 ± 0.4[b]
n-3 PUFA			
18:4n-3	0.3 ± 0.2[a]	0.5 ± 0.4[a,b]	1.2 ± 0.0[b]
20:4n-3	0.0 ± 0.0[a]	0.3 ± 0.3[b]	0.8 ± 0.0[c]
20:5n-3 EPA	3.5 ± 0.8	5.9 ± 3.3	7.8 ± 1.3
22:6n-3 DHA	5.2 ± 1.7[a]	10.5 ± 6.8[a,b]	20.1 ± 3.2[b]
22:5n-3 DPA	0.4 ± 0.3[a]	1.0 ± 0.8[a]	2.75 ± 0.1[c]
Other n-3 PUFA[g]	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0
Total n-3 PUFA	9.4 ± 0.5[a]	18.1 ± 1.9 [b]	33.0 ± 0.8[c]
n-6 PUF			
18:2n-6	1.9 ± 0.1	1.6 ± 0.2	2.3 ± 0.3
20:4n-6	2.1 ± 0.5[b]	0.8 ± 0.6[a]	1.4 ± 0.1[a,b]
22:5n-6	0.0 ± 0.0	0.2 ± 0.2	0.6 ± 0.0
Other n-6 PUFA[h]	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
Total n-6 PUFA	3.9 ± 0.1	2.6 ± 0.2	4.5 ± 0.1
Other PUFA[i]	0.7 ± 0.0	0.5 ± 0.1	1.2 ± 0.0
Total PUFA	14.0 ± 0.2[a]	21.2 ± 0.8[b]	38.8 ± 0.3[c]
n-3/n-6	2.4 ± 0.3[a]	7.1 ± 1.1[b]	7.3 ± 0.4[b]
UFA/SFA	0.8 ± 0.2[a]	0.7 ± 0.3[a]	1.7 ± 0.4[b]
Lipid class			
PE	58.4 ± 9.4	31.0 ± 9.1	49.3 ± 9.1
PI	2.4 ± 0.4[a]	2.7 ± 0.0[a,b]	3.4 ± 0.1[b]
PS	1.4 ± 0.4[a]	0.7 ± 0.1[a]	2.5 ± 0.3[b]
PC	32.3 ± 11.6	27.2 ± 10.9	34.4 ± 10.1
UPL	5.6 ± 1.4[a]	38.4 ± 2.7[b]	10.4 ± 1.2[a]
Total lipid (g/kg WW)[j]	10.96 ± 1.3	9.02 ± 1.8	10.9 ± 0.9

[a,b,c]Mean values across the row not sharing a common superscript were significantly different ($P < 0.05$) as determined by Tukey–Kramer HSD; $n = 6$.
[d]Other SFA includes 15:0, 17:0, 20:0, 22:0 and 24:0.
[e]Includes 18:3n-3 (minor component).
[f]Other MUFA includes 16:1n-9, 16:1n-5, 18:1n-5, 20:1n-7, 22:1n-9, 22:1n-11, and 24:1n-9.
[g]Other n-3 PUFA include 21:5n-3, and 24:6n-3.
[h]Other n-6 PUFA include 20:2n-6, 20:3n-6, 22:4n-6, and 24:5n-6.
[i]Other PUFA include 16:2n-4, 16:3n-4, and 18:2n-9.
[j]Determined by TLC-FID. UPL, undetermined polar lipid; UFA, unsaturated FA; for other abbreviations see Table 1.

EPA and 18:4n-3 abundances at the *sn*-2 position were significantly ($P < 0.01$) higher in the tissues relative to the diet, whereas that of DPA was significantly ($P < 0.01$) lower. The percent distribution of all the FA did not show significant differences across the three tissues. However, there were significant increases ($P < 0.01$) in EPA and 18:4n-3 and a significant decrease in DPA ($P < 0.01$) in the *sn*-2 position relative to the diet.

DISCUSSION

This is the first detailed examination of the lipid profile of Atlantic salmon at an elevated temperature (19°C). We describe the effect elevated temperature has on the composition of storage and structural lipids of three key tissues in seawater Atlantic salmon. The water temperature of 19°C was considerably above the optimum although representative of environ-

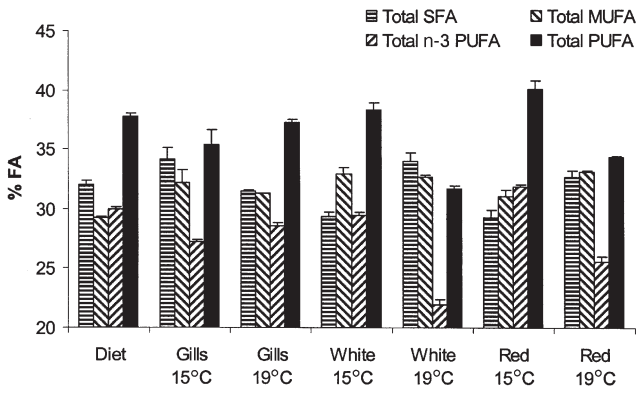

FIG. 1. Abundances of SFA, MUFA, Total ω3 PUFA and Total PUFA From the Total Lipid Extract (TLE) for the Diet, Red Muscle, White Muscle and Gills from Atlantic Salmon Salmo Salar Grown at 15 and 19°C. Values are Means with ± SE.

mental conditions now experienced by an established industry in Tasmania, Australia (23). As often observed, the FA profile of fish, particularly the storage (TAG) fraction, is strongly influenced by diet (31,32). Many studies performed at lower temperatures have shown that salmon accumulate n-3 (with a corresponding reduction in SFA) (21,22,33). We observed, in the red and white muscle, lower levels of total n-3 PUFA and total PUFA and higher SFA in the FA profile in the TLE of the 19°C fish compared with fish grown at 15°C (Fig. 1).

Environmental factors, particularly temperature, have a more significant influence on the structural lipid (PL) fraction (15,18,34,35). Elevated temperature-raised fish had higher SFA in the PLFA and lower levels of n-3, n-6, and total PUFA in the gill and white muscle. These changes suggest adaptation of the cell membrane structure has occurred, which could be explained as a response to the elevated water temperature. In other species it has been shown that there is more PUFA present and less SFA in fish that live in colder temperatures (36). Consideration of both membrane and storage lipids, including regiospecificity, allowed further examination of these adaptive changes.

Membrane lipids. Phospholipids, the membrane lipids that form the cell bi-layers, consist of two FA moieties on a glycerol backbone generally with one of five different functional phosphatidyl head groups attached. Specific FA chains can be attached at either the *sn*-1 or *sn*-2 positions. Fish phospholipids generally contain 16:0 and 18:1n-9 at the *sn*-1 position and 20:5n-3 and 22:6n-3 at the *sn*-2 position (32). This generalization is dependent on the phospholipid class and also on the particular tissue (32). In the present study 16:0 [33.4, 17.0, and 39.3%: gill, white muscle, and red muscle (G,W,R), respectively] and 18:0 (41.1, 23.6, 28.1%: G,W,R) were the major PLFA at the *sn*-1 position with 18:1 (8.9, 3.6, 7.7%: G,W,R) present at low percentages as determined by LCMS. The major PLFA at the *sn*-2 position were 22:6n-3 (46.1, 35.1, 60.7%: G,W,R), 20:5n-3 (13.6, 11.5, 14.2%: G,W,R) and 18:1 (29.2, 11.5, 8.5%: G,W,R).

In the present study the PLFA profile showed a reduced number of statistical differences between tissue FA profiles in the gills and white muscle when compared with the TLE FA profile. This observation is consistent with the PL profile being more functional (membrane structure role) and therefore more adaptive to environmental change and also less influenced by diet. The PLFA profile is also very dependent on the particular tissue analyzed.

Phospholipids generally contain high levels of 16:0, 18:1n-9, 20:5n-3, and 22:6n-3 as principal FA in the membrane bilayer (32). The PLFA fractions from the present study on Atlantic salmon contained these principal FA, as well as an enhanced percentage of 18:0 with a significantly lower percentage ($P > 0.01$) of 18:2n-6 compared with the diet. Significantly lower percentages ($P > 0.05$) of n-3, n-6, and total PUFA occurred in the gills and white muscle in the PLFA compared with red muscle and the diet (Table 3). The relative percentage of DHA was lower in the gill (5.2%) and in white muscle (10.5%), but was significantly higher ($P > 0.05$) in red muscle (20.1%). In the gill, the percentages of n-3, n-6, and total PUFA were lower, leading to higher percentages of 16:0, 18:1, and 18:0 FA, which are important pulmonary surfactants (37). Higher percent levels of DHA in the gill TAG may be attributed to the presence of residue blood in the gill tissue. The reduction of PUFA in white muscle is most likely due to the effect of elevated temperature.

The regiospecific distribution of membrane PL cannot be determined by traditional FA analysis. ESI-RP-LCMS provides a rapid method to compare relative percentages of the individual intact lipids across tissues. There is only limited work published on phospholipid molecular species distribution in salmonoid tissues determined by tandem MS (10,38). These studies, on the distribution of individual molecular species across phospholipid species, focused on the liver (38) and head kidney (10) tissues. We examined the molecular species distribution of the gill, white and red muscle and also compared them with the diet.

The principal molecular species in muscle PC in most fish is 16:0/22:6 (9). Increased PC 16:0/22:6 tends to fluidize the membrane and reduce the temperature sensitivity of electrolyte permeation (18). PC 16:0/22:6 is the predominant lipid in the PC fraction of the muscle tissues of Atlantic salmon, but not in the gills. The PI fraction was relatively high in 18:0 and low in PUFA, which was predominantly 20:4n-6; this has previously been observed in fish and was suggested as being due to a role in eicosanoid metabolism (9).

Studies on the molecular diversity of wild Atlantic cod muscle showed that SFA/PUFA and MUFA/PUFA dominated, especially PC 16:0/20:5, PC 16:0/22:6, PE 16:0/22:6, and PE 18:1/22:6 (8). The PUFA/PUFA totaled between 21 and 38% in the PC and PE fractions for cod (8). Our results showed very little PUFA/PUFA, 0.5% PC, 1.8% PE, and 4% total for white muscle and 2.7% PC, 4.7% PE, and 8.4% total in red muscle. This finding is consistent with the elevated water temperature and lower PUFA percentages noted earlier for the FAME profiles. The SFA/PUFA species dominated (42.7–64.2%) throughout the tissues, with the gill showing low (28%) levels of SFA/MUFA.

TABLE 4
Composition[a] of the Polar Lipid Fraction of White and Red Muscle and Gill Tissue of Atlantic Salmon (*Salmo salar)* Fed Commercial Formulated Diet When Held at 19°C

	Percent composition		
	Gills	White muscle	Red muscle
PC			
16:0/18:1	10.2 ± 2.4	6.8 ± 2.1	3.9 ± 0.9
14:0/22:6	0.7 ± 0.2	1.0 ± 0.6	1.6 ± 0.4
16:0/20:5	4.4 ± 1.2	4.7 ± 0.6	4.9 ± 1.1
16:1/22:6	0.9 ± 0.2	0.5 ± 0.4	0.8 ± 0.1
16:0/22:6	7.0 ± 1.7	8.2 ± 1.5	11.6 ± 3.1
16:0/22:5	2.8 ± 0.8	1.9 ± 0.5	3.1 ± 0.8
18:1/22:6	1.3 ± 0.3	0.7 ± 0.2	1.7 ± 0.5
18:0/22:6	0.8 ± 0.2	0.8 ± 0.5	0.5 ± 0.1
20:5/22:6	0.3 ± 0.1	0.5 ± 0.0	1.7 ± 0.4
22:6/22:6	0.5 ± 0.1	tr ± 0.0	1.0 ± 0.2
18:0/18:1	3.1 ± 0.8	1.2 ± 1.0	1.4 ± 0.2
15:0/22:6	0.4 ± 0.1	0.4 ± 0.3	2.1 ± 0.1
PC total	32.3	27.2	34.4
SFA/MUFA[b]	13.3	8.0	5.3
SFA/PUFA[c]	16.0	17.0	23.8
MUFA/PUFA[d]	2.2	1.2	2.6
PUFA/PUFA[e]	0.8	0.5	2.7
PE			
14:0/22:6	tr ± 0.0	0.3 ± 0.1	0.6 ± 0.1
16:0/20:5	1.1 ± 0.2	0.9 ± 0.2	1.7 ± 0.3
18:1/18:1	1.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.1
18:0/18:1	14.7 ± 2.9	3.3 ± 0.5	2.8 ± 0.6
18:2/20:5	0.2 ± 0.0	0.3 ± 0.1	0.6 ± 0.1
16:0/22:6	6.0 ± 1.5	4.9 ± 1.3	12.9 ± 3.3
16:0/22:5	1.1 ± 0.3	tr ± 0.0	tr ± 0.0
18:0/20:5	7.8 ± 2.2	5.0 ± 1.2	6.4 ± 1.1
18:2/22:6	1.4 ± 0.2	0.6 ± 0.2	1.0 ± 0.3
18:1/22:6	6.2 ± 1.7	2.6 ± 0.4	5.3 ± 1.5
18:0/22:6	13.5 ± 1.1	11.5 ± 3.0	13.5 ± 2.4
20:5/22:6	0.7 ± 0.2	0.4 ± 0.1	2.1 ± 0.5
22:6/22:6	4.6 ± 0.6	1.1 ± 0.2	2.0 ± 0.5
PE total	58.4	31.0	49.3
SFA/MUFA	14.7	3.3	2.8
SFA/PUFA	29.5	22.5	35.0
MUFA/MUFA[f]	1.2	0.2	0.4
MUFA/PUFA	6.2	2.6	5.3
PUFA/PUFA	5.4	1.8	4.7
PI			
16:0/18:2	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
16:0/20:5	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
16:0/20:4	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
16:0/22:6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
18:0/20:5	0.1 ± 0.0	0.6 ± 0.1	0.5 ± 0.1
18:0/20:4	1.5 ± 0.4	0.7 ± 0.2	0.8 ± 0.1
18:1/22:6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:0/22:6	0.3 ± 0.0	0.9 ± 0.2	1.6 ± 0.3
PI total	2.4	2.7	3.4
SFA/PUFA	2.3	2.6	3.3
MUFA/PUFA	0.1	0.1	0.1
PS			
16:0/22:6	0.4 ± 0.1	0.2 ± 0.0	0.7 ± 0.0
18:1/22:6	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
18:0/22:5	tr ± 0.0	0.1 ± 0.0	0.9 ± 0.2
20:1/22:6	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
18:0/22:6	0.8 ± 0.2	0.2 ± 0.0	0.5 ± 0.1
PS Total	1.4	0.7	2.5
SFA/PUFA	1.2	0.6	2.1
MUFA/PUFA	0.2	0.1	0.4

Continued

TABLE 4 Continued

	Percent composition		
	Gills	White muscle	Red muscle
Total			
SFA/MUFA	28.0	11.3	8.0
SFA/PUFA	49.0	42.7	64.2
MUFA/MUFA	1.2	0.2	0.4
MUFA/PUFA	8.6	4.0	8.4
PUFA/PUFA	6.2	2.4	7.4

[a]tr, trace amounts; $n = 4$. For other abbreviations see Table 1.
[b]Percentage of lipid with the SFA in the *sn*-1 and MUFA in the *sn*-2 position.
[c]Percentage of lipid with the SFA in the *sn*-1 and PUFA in the *sn*-2 position.
[d]Percentage of lipid with the MUFA in the *sn*-1 and PUFA in the *sn*-2 position.
[e]Percentage of lipid with the PUFA in the *sn*-1 and PUFA in the *sn*-2 position.
[f]Percentage of lipid with the MUFA in the *sn*-1 and MUFA in the *sn*-2 position.

TABLE 5
Percentage of FA in the *sn-2* Position in the TAG Fraction from Atlantic Salmon (*Salmo salar)* Tissues When Held at 19°C and a Fed Commercial Formulated Diet, as Determined by ^{13}C NMR Spectroscopy

	Diet	Gills	White muscle	Red muscle
DHA	66.6 ± 3.4	68.3 ± 0.1	62.1 ± 1.3	64.2 ± 0.2
DPA	50.8 ± 1.6^{b}	37.3 ± 0.7^{a}	37.8 ± 1.1^{a}	39.6 ± 0.2^{a}
EPA	23.4 ± 1.2^{a}	34.3 ± 1.3^{a,b}	36.0 ± 0.4^{b}	34.7 ± 1.3^{b}
20:4n-3	26.2 ± 1.8	ND	32.6 ± 2.3	35.3 ± 0.4
18:4n-3	35.0 ± 1.7^{a}	42.1 ± 0.6^{a,b}	45.3 ± 0.8^{b}	46.3 ± 0.1^{b}

[a,b,c]Mean values across the row not sharing a common superscript were significantly different ($P < 0.05$) as determined by Tukey–Kramer HSD; $n = 4$. ND, not determined; for other abbreviation see Table 1.

Storage lipids. Along with the structural membrane bi-layer function, lipids play an important role in energy storage in fish (39) in the form of TAG. Fish oil, which is predominantly TAG, is nutritionally important for the supply of n-3 LC-PUFA in the human diet. However, in salmon it is recognized that dietary TAG are hydrolyzed to FFA in the gastrointestinal tract prior to absorption. The regiospecific position of the dietary lipids is less important in fish than in mammals, including humans. It is well documented that consuming quantities of LC-PUFA in the form of TAG is nutritionally beneficial (40), with recent reports suggesting the position of the LC-PUFA on the glycerol backbone is also important in human nutrition (1). In general, fish oils have SFA and MUFA preferentially located at the *sn*-1 and *sn*-3 positions on the glycerol backbone, whereas LC-PUFA are generally preferentially located in the *sn*-2 position (32).

The original speculation that TAG structure might influence lipid absorption was based on observed differences in fat absorption by infants fed breast or formulated milk (3). It was shown that palmitate (16:0) in the *sn*-2 position is generally not hydrolyzed by pancreatic lipase, and the remaining MAG was well absorbed. However, when palmitate was in the *sn*-1 and *sn*-3 positions, it was hydrolyzed to form FFA, which were poorly absorbed (3). The same argument could be used for the delivery of LC-PUFA in human diet. LC-PUFA of TAG from Atlantic salmon are largely at the *sn*-2 position and are not hydrolyzed by pancreatic lipases and therefore can be absorbed into the intestinal mucosal cells of the small intestine.

It has also been shown that in whale oil EPA and DHA in the *sn*-1 and *sn*-3 positions showed a steric hindrance to pancreatic lipase hydrolysis, which may decrease their intestinal absorption (41). Therefore, fish oil with large quantities of LC-PUFA in the *sn*-2 position will be nutritionally more desirable since they are at the preferred position for absorption into the body.

The effect of regiospecific distribution of the TAG molecule on absorption and metabolism has not been thoroughly investigated in fish oils due to the wide diversity of molecular species. There have been few studies showing the relative percentages of EPA and DHA across the glycerol backbone in marine oils. Available results include fish oil (DHA 79% and EPA 53%, *sn*-2 position) (42); salmon oil (DHA 72.6% and EPA 37.8%, *sn*-2) (43); this study (DHA 62.1–68.3% and EPA 34.2–36.3%, *sn*-2), and cod liver oil (DHA 74.4% and EPA 39.7%, *sn*-2) (43). Seal oil shows contrasting regiospecificity (DHA 4.4% and EPA 4.4%, *sn*-2) (42).

There was no significant difference in the regiospecific composition of TAG across the three tissues in Atlantic salmon in the present study. The majority of DHA for all tissues is at the *sn*-2 position (62.1–68.3%), which is slightly lower compared with the 15°C fish (68.4–69.7%) and other literature for salmon oil (72.6%) (43). Our results suggest a reduced percentage of DHA at the *sn*-2 position at elevated temperature, but further work is needed to ascertain this effect. EPA showed a significant increase ($P < 0.01$) in percentage at the *sn*-2 position (34.7–36.0%) compared with the diet (23.4%). The percentages of EPA at the *sn*-2 position are comparable with results

from previous work on Atlantic salmon (44). Interestingly, the proportions of 18:4n-3 at the *sn*-2 position (42.1–46.3%) significantly ($P < 0.05$) increased compared with the diet (35.0%). The significant ($P < 0.05$) changes observed in the regiospecific distribution of DPA, 18:4n-3, and EPA, in salmon tissues compared with diet, showed that this distribution is not just a reflection of diet. Further biosynthesis or some adaptive changes have taken place in the storage of lipids as TAG in Atlantic salmon.

Temperature effects. Temperature has a major influence on the membrane and storage lipids of ectothermic animals such as Atlantic salmon, which need to adapt to seasonal and occasional abrupt changes in environmental temperature (15,17,18,31). To counteract these changes, membrane lipids may adapt in several ways: by altering the unsaturation and chain length of the FA (45); by changing the distribution of FA within the phospholipid molecules (33,46); and by altering the composition of the polar head group of the phospholipids (16,47). Gill FA profiles have been shown to vary with environmental temperature (48). In general, colder temperatures lead to an increase in unsaturation in gill lipids, thus maintaining its membrane fluidity (33,49).

Introducing a double bond to 18:0 changes the m.p. from about 70 to 20°C (39), with additional double bonds further reducing m.p. (22:6n-3, –45°C; 20:5n-3, –52°C) (39). By exploiting the diversity of the lipid structure, Atlantic salmon changes its membranes to provide physical properties to suit the environmental temperature. Since different mixtures of phospholipid molecular species, which share the same overall FA composition, can display a marked difference in physical properties, remodeling of molecular species may also help fish adapt to differences in temperature.

It is well documented that tissue FA composition of salmon, especially muscle tissue, reflects closely the FA composition of their diet (31–33,50–52). In the present study of salmon grown at 19°C, we observed significant differences between diet and tissue profiles. A significantly higher ($P < 0.05$) percentage of 16:0 occurred in white muscle, with a significantly ($P < 0.05$) higher percentage of 18:0 also observed in white muscle as well as the gills. The major differences were a significantly ($P < 0.05$) lower percentage of PUFA, in particular EPA, across all tissues compared with the diet. It has been shown that LC-PUFA, especially DHA, showed preferential deposition and retention at lower temperatures (5–15°C), regardless of the concentration present in diet (33,50,51). Comparing these results with those for fish grown at 15°C (Table 2) shows the higher temperature increased the percentage of SFA while reducing total PUFA and n-3 PUFA, particularly DHA (Fig 1). These findings indicate that there was a major adaptation of the total body lipid that was due to the higher temperature. In our study, at the elevated water temperature, there were also significantly ($P < 0.05$) lower percentages of n-3 PUFA and total PUFA in white muscle tissue. It is presently unclear and remains to be validated whether the PUFA have been directed preferentially toward metabolism, presumably for energy production, rather than to storage in muscle tissue in the form of TAG.

Cold climate has been associated with an increase in the proportions of PE and decreased proportions of PC in fish (46,49). Our results show PE was the major phospholipid in Atlantic salmon grown at 19°C. The ratio of UFA/SFA provides an assessment of the proportion of both MUFA and PUFA to SFA, and the ratio will be unchanged if MUFA replace PUFA (22). At lower temperatures, there is an accumulation of UFA and a reduction of SFA, which leads to an increase in the UFA/SFA ratio (22). As mean water temperature increases, a reduction in the UFA/SFA ratio for Atlantic salmon occurs at 10°C (4.0 UFA/SFA ratio) (35), at 11°C (3.4) (50), and at 12°C (2.5) (53). A trial looking at dietary lipids at two temperatures (2 and 8°C) showed a high UFA/SFA ratio for the muscle fraction where the diet was most like a commercial diet (high-fat fish oil). The trial separated both the polar and neutral lipids with UFA/SFA being 7.1 at 2°C and 7.0 at 8°C for PL and 5.9 at 2°C and 5.3 at 8°C for the neutral lipids (22). Our 15°C fish had a higher UFA/SFA ratio (2.4) in the red and white muscle, which is due to an accumulation of UFA (especially PUFA) in the muscle at the lower temperatures. Our results for fish grown at an elevated temperature are consistent with these patterns with UFA/SFA ratios of 0.7–1.6 observed for the PL and 1.9–2.2 for the TLE, the latter also largely neutral (TAG) lipid (81–94% of TLE). At 19°C a significant ($P < 0.05$) decrease in UFA/SFA occurred in the white muscle for the TLE fraction, with a highly significant ($P < 0.05$) decrease in the white muscle and gill tissues also occurring in the PL.

A study looking at Atlantic salmon cell culture and the effect on phospholipid class and its metabolism showed that temperature significantly affected the incorporation of PUFA in a phospholipid class (49). There was higher PUFA incorporation into PC at 10°C than at 22°C, clearly showing temperature to have an important consequence for membrane structure and possibly for function (49). In cell culture there was an increase in levels of PUFA, altered distribution of PUFA within the phospholipid classes, and decreased metabolism of C_{18} PUFA for cells grown at 10°C compared with those at 22°C (49). It has also been shown, in the marine dinoflagellate *Crypthecodinium cohnii* grown at different temperatures (16–27°C), that at the lower temperature there was a decrease in SAT/SAT and an increase in SAT/PUFA species (14).

On average, there is more PUFA present and less SFA in species of fish that live in colder regions (36,49). High levels of SFA in diets have been associated with poor human health, and recommendations have been made to reduce SFA levels in diets (54). It is also well known that eating foods containing MUFA and specific n-3 and n-6 LC-PUFA have associated health benefits (55–58). As a source of these MUFA and PUFA, fish and especially fish oil have been traditionally the major resource. Increased SFA in white muscle has implications for the salmon aquaculture industry in regions where water can reach temperatures of 19°C and beyond in the warmer months. The results also suggest a reduction in the need for dietary PUFA over these warmer months. Economically this would be advantageous to the aquaculture industry due to the high cost involved with supplying LC-PUFA-rich fish oil in the diet. Con-

versely, if harvest occurs over these warmer months, the percentage of LC-PUFA in these fish will be lower and will reduce the nutritional quality of the product with respect to n-3 LC-PUFA content. The precise physiological significance of the phospholipid distribution and how incorporation of these results into feed formulation may occur remains to be developed. With new methods such as LCMS and ^{13}C NMR becoming more accessible and automated, regiospecific analysis can be routinely applied in investigations of marine oils. Such information, as it becomes available, will ultimately provide improved understanding on the distribution, function, and bioactivity of LC-PUFA-containing oils.

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Convenient and Efficient Syntheses of 4-Hydroxy-2(*E*)-nonenal and 4-Oxo-2(*E*)-nonenal

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ABSTRACT: Lipid peroxidation products 4-hydroxy-2(*E*)-nonenal (HNE) and 4-oxo-2(*E*)-nonenal (ONE) were conveniently synthesized using Wittig and Horner-Wardsworth-Emmons (HWE) reaction. Wittig or HWE reaction between an easily prepared phosphorane or phosphonate with glyoxal dimethyl acetal gave a protected 4-oxo-2(*E*)-nonenal. Hydrolysis gave 4-oxo-2(*E*)-nonenal, whereas reduction followed by hydrolysis gave 4-hydroxy-2(*E*)-nonenal.

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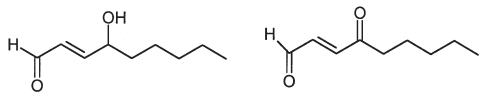
Lipid peroxidation in biological systems produce many degradation products including 4-hydroxy-2(*E*)-nonenal (HNE) **1** and 4-oxo-2(*E*)-nonenal (ONE) **2** (1,2). These are well known for their genotoxic and cytotoxic effects (1,3–5). The extreme cytotoxicity of HNE is a manifestation of its ability to inhibit several enzymes like protein kinase C, adenylate cyclase, glyceraldehyde-3-phosphate dehydrogenase, and others through modification of nucleophilic amino acid residues (6–9). HNE has been shown to induce apoptosis (10,11) and to cause changes in global gene expression (12). HNE readily modifies nucleophilic protein side chains and DNA bases and its bifunctionality results in protein cross-linking (13,14).



1 **2**

There is increasing evidence that HNE is casually involved in most of the pathophysiological effects associated with inflammatory and oxidative stress in cells and tissues (15,16). HNE is thought to play a significant role in neurodegenerative diseases, in particular Alzheimer's disease (17–20) and Parkinson's disease (21–23). In addition to studies on its toxicity, protein and DNA adducts of HNE are commonly used as a biomarker for the occurrence and/or the extent of lipid peroxidation (24).

ONE is an even more potent electrophile and therefore more neurotoxic and highly protein reactive (5). Thus, both HNE and ONE participate in the formation of DNA adducts, making it possible to assess the role of lipid peroxidation in mutagenesis and carcinogenesis (2).

We required substantial amounts of these compounds for our study. Previous procedures for HNE preparation employed variations in the Grignard reaction (25–30), isomerization of 2-yne-1,4-diols with ruthenium catalyst (31), a method via 1,3-bis(methylthio)allyl-lithium (32), a sequence of addition/elimination/substitution reaction performed on the corresponding saturated aldehyde (33,34), epoxidation of 3(*Z*)-nonenol, oxidation, and isomerization (35). A method for chiral synthesis for HNE is also reported (36) which involves Sharpless epoxidation, reaction with an unstable Wittig reagent followed by isomerization (36). The methods for the synthesis of ONE employed lithiation of furan using *n*-BuLi, alkylation using *n*-pentyliodide, followed by either methoxylation using bromine in methanol, ring cleavage and isomerization (37), or oxidative ring opening of furan using PCC (38). We report herein a simple and convenient method for the preparation of HNE **1** and ONE **2.**

EXPERIMENTAL PROCEDURES

^{1}H NMR and ^{13}C NMR spectra were recorded on 300-MHz and 400-MHz Bruker instruments. The multiplicities of carbon signals were obtained from a distortionless enhancement by polarization transfer (DEPT) experiment. Chemical shifts (ppm) are relative to the internal standard Me_4Si (0 ppm). IR spectra were recorded on a Shimadzu FT-IR spectrophotometer (KBr pellet or neat sample). TLC was performed on silica gel G (13% $CaSO_4$ as binder). Triethylphosphonoacetate was purchased from Aldrich Chemical Company (Sigma-Aldrich, Mumbai, India), and Glyoxal dimethyl acetal was purchased from Lancaster (Lancaster, A Clariant Group Company, Chennai, India).

The stable Wittig reagent ethyl (triphenylphosphoranyliden)acetate **3a** was acylated with hexanoyl chloride to provide acylated Wittig reagent **4a** (39). The Wittig reagent **4a** on decarboxylative hydrolysis provided acyl Wittig reagent 1-(triphenylphosphoranylidene)-2-heptanone **5a** (Scheme 1).

The diethyl-2-oxoheptylphosphonate **5b** was prepared by acylating triethyl phosphonoacetate to obtain **4b**. Hydrolysis and decarboxylation of **4b** gave phosphonate **5b** (Scheme 2).

On condensation of **5a** or the anion of phosphonate **5b** with glyoxal dimethyl acetal, an α,β-unsaturated ketone **6** was formed, which on deprotection gave ONE **2** and after reduction followed by deprotection gave HNE **1** (Scheme 3).

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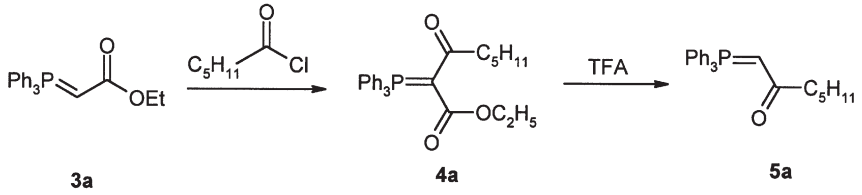
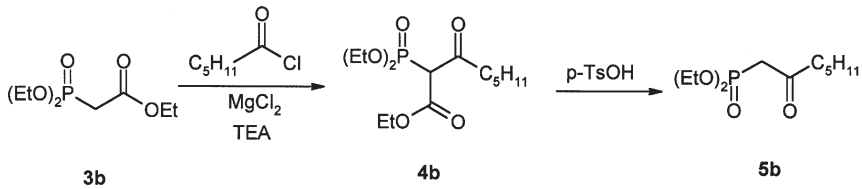
Abbreviations: DEPT, distortionless enhancement by polarization transfer; HNE, 4-hydroxy-2(*E*)-nonenal; ONE, 4-oxo-2(*E*)-nonenal.



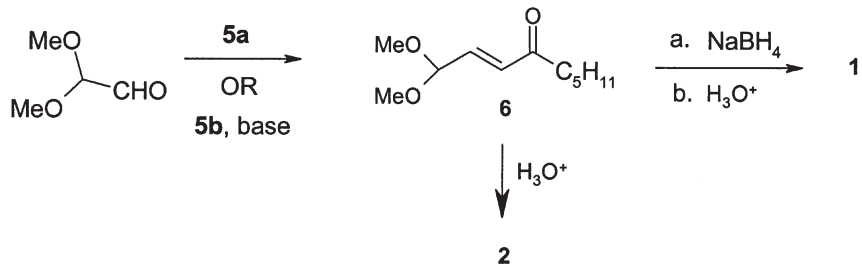
3a **4a** **5a**

SCHEME 1



3b **4b** **5b**

SCHEME 2



SCHEME 3

*Preparation of Wittig reagent (**5a**).* The stable Wittig reagent **3a**, 5.2 g (14.8 mmol), dissolved in toluene (15 mL) was added to hexanoyl chloride 1 g (7.4 mmol). The yellow solid obtained, after stirring for 5.0 h at room temperature, was filtered. The product obtained after concentration of the filtrate under reduced pressure was 3.1 g (**4a**, yield 93.5%). IR (KBr): ν_{max} 2949.16, 2927.94, 2850.00, 1662.64, 1556.55, 1103.28, 752.20, 692.40 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ_{ppm} 0.63 (t, 3H, J = 7.1 Hz, CH_3), 0.83 (t, 3H, J = 6.8 Hz, CH_3), 1.28 (m, 4H, CH_2), 1.59 (m, 2H, CH_2), 2.84 (t, 2H, J = 7.6 Hz, CH_2CO), 3.70 (q, 2H, J = 7.1 Hz, CH_2), 7.40–7.65 (m, 15H, ArH). A solution containing 1.70 g (3.8 mmol) of phosphorane **4a** in a mixture of trifluoroacetic acid (15 mL) and water (2.5 mL) was heated under reflux for 6.0 h. The reaction mixture was then poured onto ice and basified with 2% sodium bicarbonate solution. This was followed by extraction with diethyl ether (3 × 10 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography using ethyl acetate/hexanes (1:5 vol/vol) to obtain 0.71 g (yield 50%) of **5a**. IR (KBr): ν_{max} 2927.56, 1537.25, 1436.00, 1392.31, 1195.14, 751.21, 749.51, 719.45 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz) δ_{ppm} 0.80 (t, 3H, J = 7.0 Hz, CH_3), 1.25 (m, 4H, 2 × CH_2) 1.58 (m, 2H, CH_2), 2.22 (t, 2H, J = 7.1 Hz, CH_2CO), 4.37 (br s, 1H, $CH{=}PPh_3$), 7.2–7.6 (m, 15H, ArH).

*Preparation of **5b**.* A solution of hexanoyl chloride 5.8 g (43.12 mmol) in dry toluene (21 mL) was slowly added over a period of 2.0 h. to a cold solution (5°C) of triethyl phosphonoacetate (9.7 g, 43.12 mmol), $MgCl_2$ (4.1 g, 43.12 mmol), and triethylamine (13.06 g, 129.4 mmol) in toluene (125 mL). The reaction was stirred for 6.0 h at room temperature; the reaction was then quenched into an ice-cold solution of 10% H_2SO_4 (125 mL). The two layers were separated and the aqueous layer was extracted with ethyl acetate (4 × 25 mL). The combined organic extracts were dried over Na_2SO_4. The product **4b** obtained after filtration and concentration of the organic layer was 13.3 g (yield 96.18%). The compound was judged as pure by observation of a single spot on TLC (ethyl acetate/hexanes 2:3 vol/vol). A solution of **4b** (13.3 g, 43.4 mmol) and *p*-toluenesulfonic acid (0.21 g, 1.2 mmol) in H_2O (175 mL) was heated under reflux for 3.0 h. The reaction mixture was extracted using diethyl ether (3 × 50 mL). The combined organic extracts were dried over Na_2SO_4. The product obtained after filtration and solvent evaporation under reduced pressure was purified by silica gel column chromatography (ethyl acetate/hexanes, 1:5 vol/vol) to obtain 8.1 g (yield 74.7%) of **5b**. IR (neat): ν_{max} 2958.00, 2932.00, 1708.04, 1254.75, 1025.21, 964.45 cm^{-1}. ^1H-NMR ($CDCl_3$, 300 MHz) δ_{ppm} 0.81 (t, 3H, J = 6.9 Hz, CH_3), 1.20 (m, 4H, 2 × CH_2), 1.27 (t, 6H, J = 7.0 Hz, OCH_2CH_3), 1.52 (m, 2H, CH_2CH_2CO), 2.55 (t, 2H, J = 6.9 Hz,

CH_2CO), 3.00 (d, 2H, J = 22.8 Hz, $COCH_2P$), 4.06 (q, 2H, J = 7.0 Hz, OCH_2CH_3), 4.11 (q, 2H, J = 7.0 Hz, OCH_2CH_3).

*Preparation of α,β-unsaturated ketone (**6**) from **5a.*** A solution containing glyoxal dimethyl acetal 4.0 g (10.7 mmol) and Wittig reagent **5a** 1.8 g (11.7 mmol) in methanol (20 mL) was heated at reflux for 3.0 h. The solvent was removed under reduced pressure and the residue was chromatographed (column chromatography, hexanes), giving 1.26 g (yield 59.03%) of **6** as an oil. IR (neat): ν_{max} 2956.87, 2933.73, 1709.00, 1680.00, 1130.29, 1058.92, 979.84 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ_{ppm} 0.8 (t, 3H, J = 6.75 Hz, CH_3), 1.2 (m, 4H, 2 × CH_2), 1.56 (m, 2H, CH_2), 2.5 (t, 2H, J = 7.35 Hz, CH_2CO), 3.2 (s, 6H, OCH_3), 4.9 (d, 1H, J = 4.2 Hz, C*H*$(OCH_3)_2$), 6.3 (d, 1H, J = 16.2 Hz, =C*H*CO), 6.55 (dd, 1H, J = 16.2 & 4.2 Hz, CH=C*H*-CH). ^{13}C-NMR ($CDCl_3$) δ_{ppm} 14.20 (C9), 22.41 (C8), 23.71 (C7), 31.40 (C6), 40.65 (C5), 52.90 (C1), 101.10 (OCH_3), 132.10 (C2), 139.90 (C3), 200.46 (C4).

*Preparation of α,β-unsaturated ketone (**6**) from **5b.*** A solution of 1 g (25 mmol) of NaOH in 2 mL of H_2O was added to the mixture of 0.58 g (2.3 mmol) of **5b**, 0.35 g (3.3 mmol) of glyoxal dimethyl acetal, 0.04 g of tetrabutylammonium iodide in 3 mL of dichloromethane. After 30 min of stirring at 28°C, the reaction mixture was extracted with dichloromethane (3 × 5 mL). After drying all the combined organic layers over Na_2SO_4, filtration, and concentration, the resulting residue was passed through a silica gel column (hexanes) to obtain 0.43 g (yield 92.45%) of **6**.

*Preparation of **2.*** A solution containing α,β-unsaturated ketone **6** (0.1 g, 0.5 mmol) and citric acid (0.1 g) in water (10 mL) was stirred at room temperature for 2.0 h and then extracted in chloroform (3 × 10 mL). Combined organic extracts were concentrated to obtain 0.062 g (yield 80%) of the product. IR (neat): ν_{max} 2956.87, 2933.73, 2872.00, 1701.00, 1680.00, 1350.00, 1130.00, 1058.00 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ_{ppm} 0.9 (t, 3H, J = 7.00 Hz, CH_3), 1.28 (m, 4H, 2 × CH_2) 1.61 (m, 2H, CH_2), 2.56 (t, 2H, J = 7.00 Hz, CH_2CO-), 6.35 (dd, 1H, J = 17.00 and 7.00 Hz, =CH-CHO), 6.6 (dd, 1H, J = 17.00, =CHCO), 9.78 (d, 1H, J = 7.00 Hz, CHO).

*Preparation of **2.*** To the stirred solution of **6** (0.1 g, 0.5 mmol) in dichloromethane (2 mL) was added 0.152 g of montmorillonite K-10 (40) and stirred at room temperature for 30 min. The reaction was filtered, the residue was washed with dichloromethane (2 × 5 mL), and the combined filtrate concentrated under vacuum to give 0.071 g (92%) of pure 4-ONE.

*Preparation of **1.*** Sodium borohydride 0.0208 g (0.55 mmol) was added to the solution of 0.1 g (0.5 mmol) of **6** in 5 mL absolute ethanol. After stirring the reaction for 2.0 h at 28°C, the solvent was removed under vacuum. To the residue, water (5 mL) was added and then extracted in diethyl ether (3 × 5 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum to obtain 0.097 g (96%) of the protected HNE. IR (neat): ν_{max} 3442.94 (br), 2931.80, 2858.51, 1130.29, 1053.13, 970.19 cm^{-1}.

To the stirred solution of the above protected HNE (0.0813 g, 0.4 mmol) in dichloromethane (2 mL) was added 0.123 g of montmorillonite K-10 and stirred at room temperature for 30 min. The reaction was filtered, the residue was washed with dichloromethane (2 × 5 mL), and the combined filtrate was concentrated under vacuum to give 0.064 g (83.5%) of pure HNE

IR (neat): ν_{max} 3442.94 (br), 2931.80, 2858.51, 1693.50, 975.98, 734.88. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ_{ppm} 0.91 (t, 3H, J = 7.00 Hz, CH_3), 1.33 (m, 5H, 2 × CH_2 and C*H*CHOH), 1.65 (m, 2H, C*H*$_2$CH$_2$CHOH), 2.62 (t, 1H, J = 7.1 Hz, HC*H*CHOH), 4.45 (m, 1H, C*H*OH), 6.33 (m, 1H, CHOC*H*=), 6.84 (dd, 1H, J = 4.8, J = 15.6 Hz =C*H*CHOH), 9.6 (d, 1H, J = 7.1 Hz, CHO).

RESULTS AND DICUSSION

Previously described methods (25–38) mostly require anhydrous, inert, and/or cryogenic reaction conditions, use of organometallic reagent, and are used only for the preparation of either HNE or ONE. We required large amounts of both ONE and HNE for our studies so we visualized a convergent approach. The first approach using phosphorane gives us the key intermediate **6** in slightly low yield (59%) due to its decomposition on silica gel column. The latter phosphonate approach (Horner-Wardsworth-Emmons) was the most efficient method giving **6** in 92.5% yield. ONE was obtained from **6** using citric acid for hydrolysis in 74% overall yield. Use of montmorillonite K-10 (40) provided ONE in 83% overall yield. HNE was obtained from **6** by reduction with $NaBH_4$ followed by deprotection using montmorillonite K-10 in 80.7% overall yield. Both our methods are advantageous over the earlier methods in terms of overall yield, simplicity of procedures, availability of the chemicals, and also are easily scalable. In both methods the unsaturated ketone **6** has exclusive *E* geometry.

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REVIEW

Anticancer Activity of Natural and Synthetic Acetylenic Lipids

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ABSTRACT: This review is a comprehensive survey of acetylenic lipids and their derivatives, obtained from living organisms, that have anticancer activity. Acetylenic metabolites belong to a class of molecules containing triple bond(s). They are found in plants, fungi, microorganisms, and marine invertebrates. Although acetylenes are common as components of terrestrial plants, fungi, and bacteria, it is only within the last 30 years that biologically active polyacetylenes having unusual structural features have been reported from plants, cyanobacteria, algae, invertebrates, and other sources. Naturally occurring aquatic acetylenes are of particular interest since many of them display important biological activities and possess antitumor, antibacterial, antimicrobial, antifouling, antifungal, pesticidal, phototoxic, HIV-inhibitory, and immunosuppressive properties. There is no doubt that they are of great interest, especially for the medicinal and/or pharmaceutical industries. This review presents structures and describes cytotoxic and anticancer activities only for more than 300 acetylenic lipids and their derivatives isolated from living organisms.

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Natural acetylenic lipids and their derivatives have been isolated from a wide variety of plant species, cultures of higher fungi, and marine invertebrates (1–8). Many of them display important biological activities, including antitumor, antibacterial, antimicrobial, antifungal, phototoxic, and other chemical and medicinal properties (9,10). Currently, about half of all prescribed medicines are extracted or derived from terrestrial plants and microorganisms. Most of the synthetic drugs, it should be noted, were originally inspired by novel compounds discovered in terrestrial organisms (11–13). Plants have been used worldwide since antiquity for treatment of various human ailments. Their use is still quite prevalent in developing countries in the form of traditional/folkloric medicine.

Intensive chemical and pharmacological studies during the last five decades have led in many cases to validation of traditional claims and facilitated identification of the traditional medicinal plants and of their active principles. Biological activities of acetylenes and related compounds from higher plants have been studied intensively in recent years, and their activity in various organisms is now well documented. More than 1000 acetylenic metabolites have been isolated from plants, fungi, microorganisms, and marine invertebrates and identified (1–5,14–17).

In the past several decades, natural acetylenic compounds have been isolated from a wide variety of macro- and microalgal species, freshwater and marine cyanobacteria, and other aquatic organisms. Extensive pharmacological screening performed on aquatic species resulted in discovery of novel antitumor agents (1,5,6). The purpose of this review is to summarize antitumor and cytotoxic properties of 318 terrestrial and aquatic acetylenic natural products, belonging to diverse structural classes, including aliphatic and cyclic polyketides, terpenes, steroids, lipids, carotenoids, and peptides. The species yielding these bioactive compounds comprise a taxonomically diverse group of terrestrial and aquatic organisms (1,18–20).

ACETYLENIC METABOLITES FROM PLANT SPECIES

Two polyacetylenes, heptadeca-3-oxo-4,6-diyne-9,10-diol (panaxacol **1**) and its dihydro derivative (dihydropanaxacol **2**) were isolated from callus of the *Panax ginseng*. Both compounds inhibited the growth of Yoshida sarcoma cells in tissue culture, and have been synthesized (21–24). Panaxyne (**3**) (tetradeca-13-ene-1,3-diyne-6,7-diol) was isolated from the Korean red ginseng (25,26). The ED_{50} (dose that is effective in 50% of test subjects) value of panaxyne for cytotoxicity against L-1210 cell was 11 μg/mL. The cytotoxic activity of the compound was lower than that manifested by other ginseng polyynes, which was presumably due to a lack in the panaxyne molecule of an essential structural part (hept-1-en-4,6-diyne-3-ol). Another polyacetylene compound isolated from *P. ginseng* possessed a diyne-ene and an epoxy moiety (27). Tetradeca-13-ene-1,3-diyne-6,7-epoxide (panaxyne epoxide **4**) revealed cytotoxic activity against L-1210 cells (27).

The content of some polyacetylenes in *P. ginseng* is quite high. Thus, panaxydol (**5**), panaxynol (falcarinol) (**6**), and panaxytriol (**7**) are present in red ginseng powder at concentrations of 297, 250, and 320 μg/g, respectively. The antitumor alcohols panaxydol and panaxynol, isolated from a powder of the root of P. ginseng, inhibited the growth of various kinds of cultured tumor cell lines (28–32).

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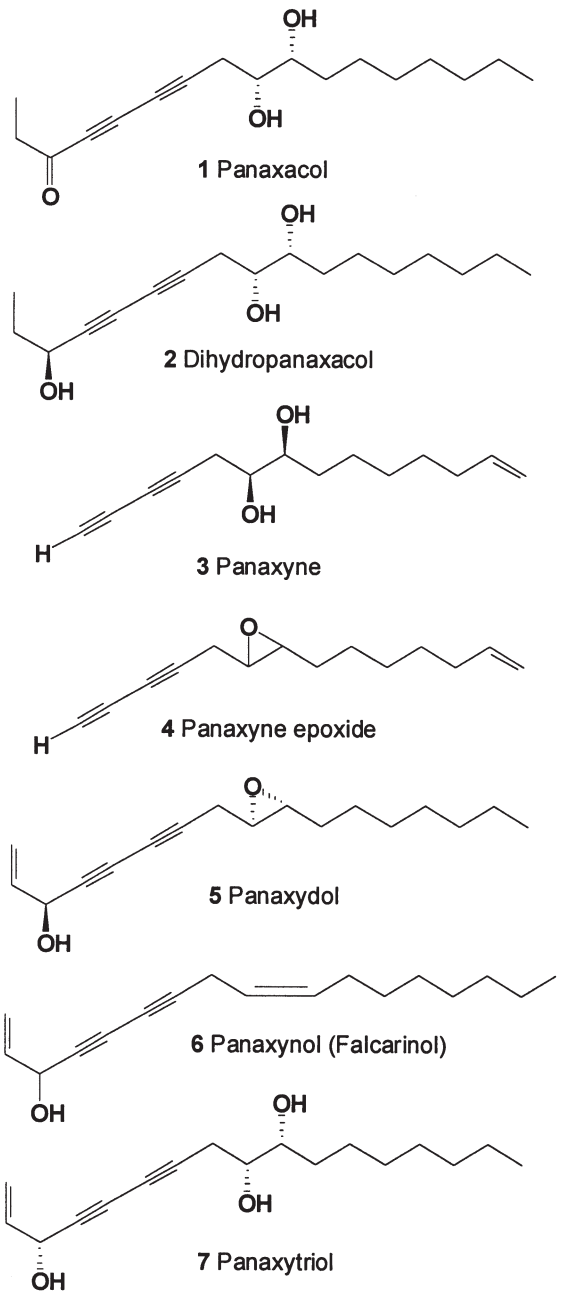
Abbreviations: A549, lung carcinoma cells; COX, cyclo-oxygenase; ED_{50}, dose that is effective in 50% of test subjects; HeLa, cervical cancer cells; IC_{50}, concentration that is inhibitory to 50% of the test subjects; KB, KB carcinoma cells; L-1210, murine leukemic cells; LC_{50}, concentration lethal to test animals in a given time, usually 4 h; LOX, lipoxygenase; MK-1, human gastric adenocarcinoma; P388, murine lymphocytic leukemia; PA, phosphatidic acid; PC, phosphatidylcholine; PQ, panaquinquecol.



The α-cyclodextrin complexes of **5** and **6** inhibited growth of various kinds of cultured cell lines in a concentration-dependent fashion. Their inhibitory activity was much stronger against malignant cells than against normal cells. ATPase activities of cells from Sarcoma 180 and rat liver were slightly inhibited by panaxydol (**5**). Activities of 5′-nucleotidase and succinate cytochrome C reductase in Sarcoma 180 cells and in rat liver, respectively, were significantly inhibited in a dose-dependent manner by panaxynol (**6**) (33).

To investigate the cytotoxic mechanism of polyacetylenes against cancer cell lines, the effects of these compounds were tested on cell membranes (rat erythrocytes and murine leukemic L-1210 cells) as well as on artificial lipid bilayers (34). Panaxydol and panaxynol caused concentration-dependent hemolysis, whereas panaxytriol produced no lysis. With liposomes composed of phosphatidylcholine (PC) and phosphatidic acid (PA), all three polyacetylenes suppressed osmotic behavior to the same degree. Panaxytriol gave its least suppression with liposomes of PC, PA, and cholesterol. Electron microscopic observations demonstrated that panaxydol caused nonspecific injury to L-1210 cells by affecting the cell membrane, nuclear envelope, and mitochondria. The cytotoxicity of polyacetylenes may thus be related to membrane damage. A relatively polar panaxytriol had little effect on the cholesterol of lipid bilayers. Panaxytriol (**7**), from *P. ginseng*, showed antiproliferative effects against several kinds of tumor cells, mitomycin C (MMC), and a human gastric carcinoma cell line, MK-1 (35). Panaxytriol also inhibits human breast carcinoma cells (Breast M25-SF, 180 mM) *in vitro* and the growth of B16 melanoma transplanted into mice (36).

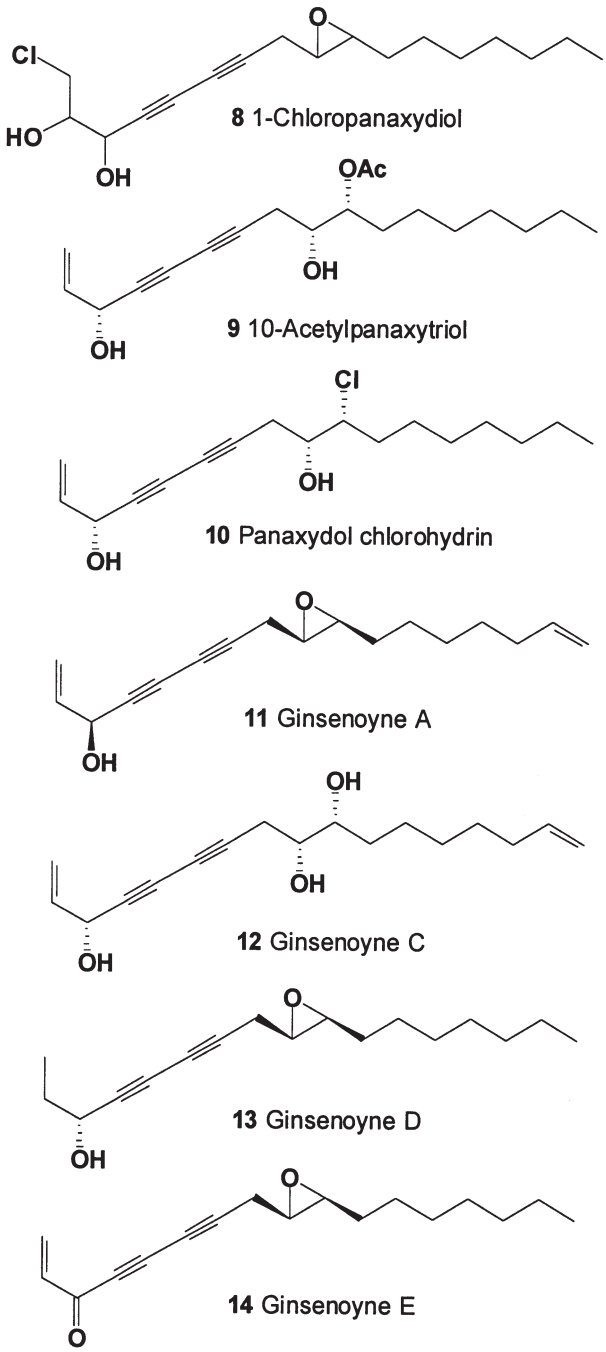
Panaxydol (**5**), panaxynol (falcarinol) (**6**), and panaxytriol (**7**) inhibited the synthesis of DNA, RNA, and protein in lymphoid leukemia L-1210 cells incubated with the drugs for 4–16 h (25). Falcarinol showed anti-inflammatory activity; had a marginal effect on cyclo-oxygenase (COX) activities [IC_{50} (concentration that is inhibitory to 50% of the test subjects) values > 100 μM]; and inhibited 5-lipoxygenase (5-LOX: IC_{50}, 2 μM), two isoforms of 12-LOX (leukocyte-type, 1 μM and platelet-type, 67 μM), and 15-LOX (4 μM) (37–39).

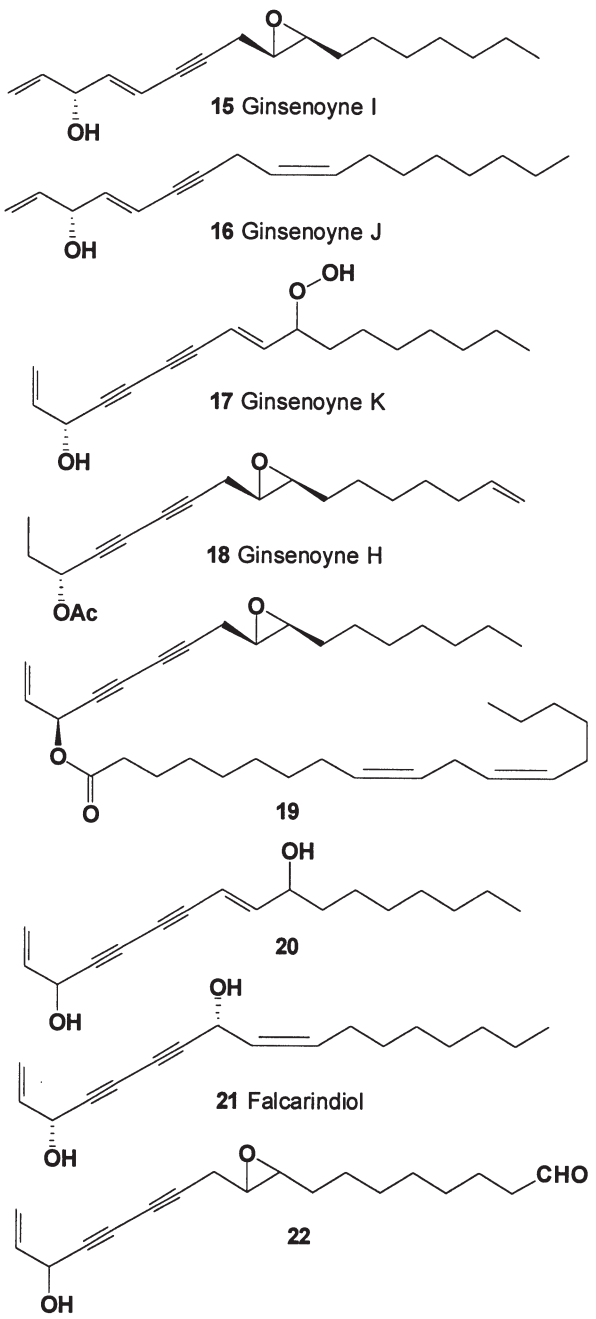
Panaxytriol (**7**), from red ginseng *(P. ginseng*), was studied to detect its effects on the growth and cell cycle of tumor cell lines (40,41). It showed both significant cytotoxicity toward and inhibition of DNA syntheses in various tumor cells. For P388D1, a mouse lymphoma cell line, IC_{50} values for cytotoxicity and inhibition of DNA synthesis were 3.1 and 0.7 μg/mL, respectively. Also panaxytriol exhibited antiproliferative activity toward the tumor cell lines MK-1, HeLa, and B16F10 (40,41). The cytotoxic effect of panaxytriol was both time- and dose-dependent. It also induced the cell cycle arrest of P388D1 at the G2/M phase. In particular, flow cytometry data indicated that the proportion of cells arrested in the G2/M phase increased from 9 to 26 and 48%, respectively, after 24 and 36 h exposure to panaxytriol at 5 μg/mL. A similar decrease was found for the cells arrested at the G0/G1 phase.

A series of other polyacetylene compounds of ginseng origin, including chlorine-containing chloropanaxydiol (**8**) and panaxydol (**5**), were tested for cytotoxic activities in different cell and tissue cultures (42–44). Chloropanaxydiol (**8**) showed inhibitory activity against leukemia cells (L-1210) in tissue culture and exhibited fungicidal properties. The heptadeca-1-ene-4,6-diyne-3,9-diol 10-acetate [10-acetyl panaxytriol (**9**) from Korean ginseng roots] showed strong cytotoxic activity against L-1210 cells (ED_{50} = 1.2 μg/mL) (45). Acetylenic compounds **3–7** and **10–19** from the root of *P. ginseng* were tested for their cytotoxic activities on murine and human malignant cells (DT, NIH/3T3, L-1210, HeLa, T24, and MCF7 cells) *in vitro* (46). Most of them showed more potent cytotoxicity than 5-fluorouracil and cisplatin. Of the active compounds, panaxydol was found to be most efficient (IC_{50} DT cells, 0.65 μM; 3T3 cells,

1.3 μM; L-1210 cells: 0.19 μM). Compound **19** inhibited growth of L-1210 line cells at an IC_{50} of 14 μg/mL (47).

Other isolated polyacetylenic alcohols—panaxynol (**6**) and heptadeca-1,8-diene-4,6-diyne-3,10-diol (**20**) (*Saposhnikoviae radix*: Fang Feng in Chinese Medicine) and falcarindiol (**21**) (from *Peucedani radix*, Qian Hu), 9,10-epoxy-16-hydroxy-octadeca-17-ene-12,14-diyne-1-al (**22**) (from *Foeniculi fructus*, bitter fennel) as well as panaxydol **5**, panaxynol (**6**), and panaxytriol (**7**) (from *Panax ginseng)*—inhibited the growth of cells from a human gastric adenocarcinoma cell line, MK-1, in a dose-dependent manner (48–51).





Three new cytotoxic polyacetylenes, panaquinquecol-1 (PQ-1: **23**), PQ-2 (**24**), and PQ-3 (**25**), from *Panax quinquefolium* exhibited strong cytotoxic activities against leukemia cells (L-1210) in tissue culture (52). Anticancer agents panaquinquecol 4 (PQ-4) (**26**), panaquinquecol 5 PQ-5 (**27**), and panaquinquecol 6 (PQ-6) (**28**) were isolated from root extracts of *P. quinquefolium* as active ingredients and had IC_{50} values of 0.5, 10, and 0.5 μg/mL, respectively, against murine leukemia L-1210 (53). C_{17}-Polyacetylenes PQ-4 (**26**) and PQ-6 (**28**) and a C_{14}-polyacetylene PQ-5 (**27**) were isolated from dried roots of *P. quinquefolium* (54). The cytotoxic activity of C_{17}-polyacetylenes **26** and **28** against leukemia cells (L-1210)

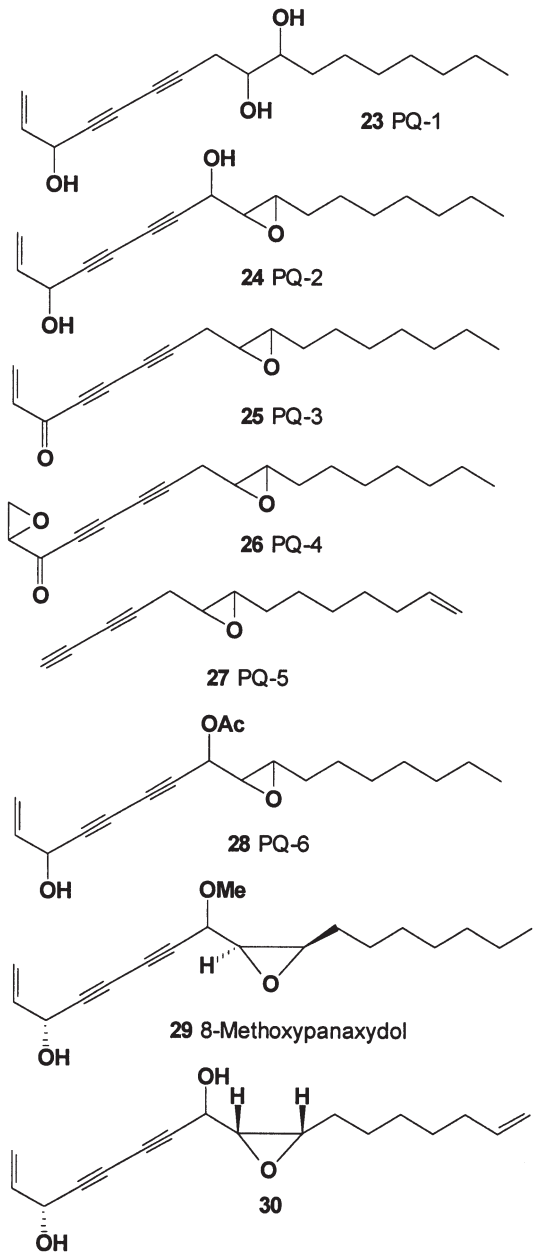
was about 20 times higher than that of the C_{14}-polyacetylene (**27**). Panaxydol (**5**), heptadeca-1,8-diene-4,6-diyne-3,10-diol (**20**), and 8-methoxy-panaxydol (**29**) from *Acanthopanax senticosus* roots seemed to induce various pro-apoptosis mechanisms in animal cells (55–62). Compound **29** was preferably used in treating leukemia.

Anticancer agents 1,16-heptadecadiene-4,6-diyne-3,9,10-triol (**12**) and compound **30,** which inhibited L-1210, Ehrlich, and HeLa cell lines with IC_{50} of 0.2, 1.3, and 2.1 μg/mL, respectively, have been isolated from Japanese ginseng (63). C_{17}- and C_{14}-polyacetylenes (**31**) and (**32**: PQ-8), from the dried roots of *P. quinquefolium*, showed strong cytotoxic activity (IC_{50} = 0.1 and 0.5 μg/mL, respectively) against leukemia cells (L-1210) in tissue culture (64). Falcarinol (**6**), falcarindiol (**21**), and falcarinolone (**34**) from the Chinese crude drug Toki (the roots of *Angelica acutiloba* var. *acutiloba*, Umbelliferae) inhibited AcOH-induced writhing in mice (65). Falcarindiol also showed antinociceptive activities in the retrograde injection test of bradykinin into a carotid artery of rats. Bioactive crepenynic acid (**33**) was obtained from crude extracts of *Crepis rubra* (66–69). Three compounds, **6**, **21**, and **34**, have also been isolated from carrots and other root crops, and their phytoprotective, cytotoxic, and allergic properties reviewed (70,71). *Dendropanax arboreus* is native to North America. It has spread from Mexico, across Central America, to northern South America and the West Indies. Its major ingredient that was responsible for *in vitro* cytotoxicity was falcarinol (**6**). Several other related compounds, including dendroarboreols A (**35**) and B (**36**), dehydrofalcarinol (**37**), dehydrofalcarindiol (**38**), and (**40**) were proven to be effective (72–74). Their cytotoxic effects are shown in Table 1.

TABLE 1
***In vitro* Cytotoxicity[a] Potencies of Different Compounds (LOX melanoma mouse; ref. 72)**

Compound	GI_{50}[b] (μg/mL)	TGI[b] (μg/mL)	LC_{50}[b] (μg/mL)
6	0.22	0.76	3.8
21	0.66	2.2	7.6
35	4.6	14.0	25.0
36	0.5	1.9	7.6
37	0.4	1.3	4.8
38	2.1	3.0	10.0
40	0.44	1.4	4.7

[a]In National Cancer Institute's 60-cell-line human tumor screening panel; data reported (molarity) are overall panel averages of quadruplicate tests, unless otherwise noted.
[b]GI_{50}, drug-treated cells' net growth is 50% of untreated controls; TGI, no net growth of drug-treated cells; LC_{50}, 50% net cell death in drug-treated cells vs. starting levels.



The active component *cis*-1,9,16-hepta-decatriene-4,6-diyne-3,8-diol (**39**) has been isolated from *Dendropanax arboreus* extract (75,76). The same compound has recently been found in the hairy root culture of *Ambrosia maritima*; it showed cytotoxic action against tumor cell lines LS174T (IC_{50}, 14.8 μg/mL), SKCO1 (IC_{50}, 13.3 μg/mL), COLO320DM (IC_{50}, 9.6 μg/mL), WIDR (IC_{50}, 10.9 μg/mL), MDA231 (IC_{50}, 37.6 μg/mL), and MCF7 (IC_{50}, 5.8 μg/mL) (77).

Acetylenic compounds **41–44**, including two chlorine-containing metabolites, **41** and **42**, are inhibitors of HL-60 cells (Table 2) and were isolated from the leaves of *Artemisia lactiflora* (Compositae, Thailand) (78–80). Compounds **44**, **46**, and **47** were evaluated for their ability to inhibit 12-LOX, and **46** and **47** showed moderate activity at 30 μg/mL. Compound **44** was tested on a series of colorectal and breast cancer cell lines, and its IC_{50} values ranged from 5.8 to 37.6 μg/mL. Compound **41** showed inhibitory activity against *Staphylococcus aureus* (ATCC) at concentrations in the range of 64–128 μg/mL.

The inhibitory effects of a diacetylenic spiroketal enol ether epoxide [AL-1 (**45**) from white mugwort, Asteraceae; *Artemisia lactiflora*] on a variety of tumor promoter-induced biological responses such as oxidative stress as well as tumor

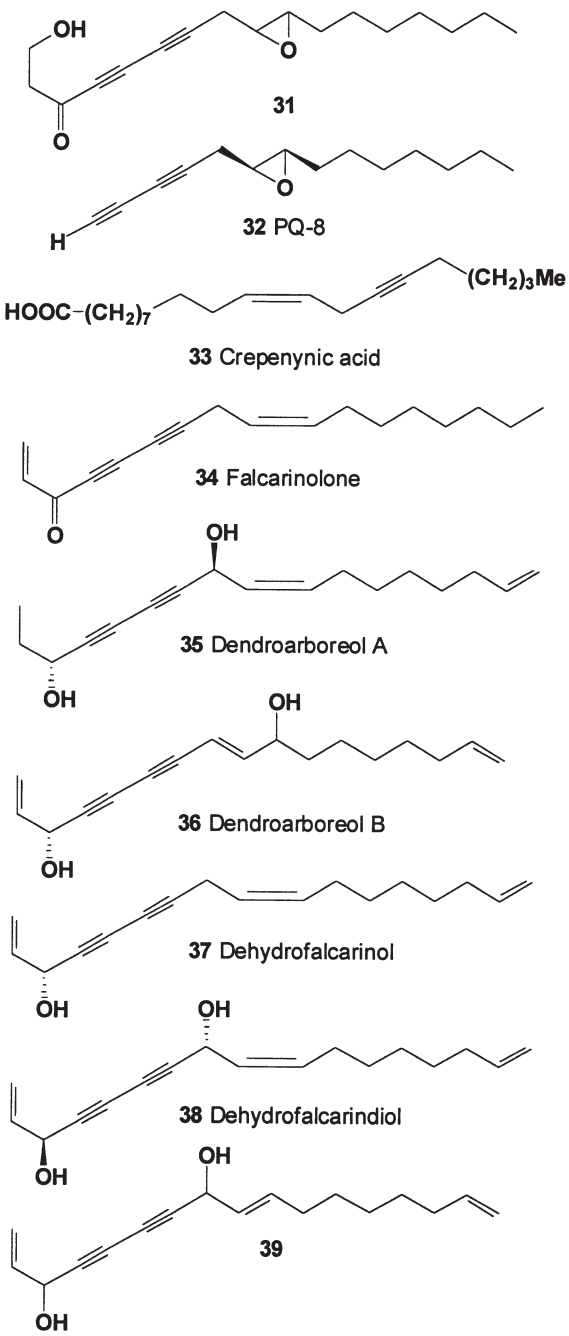
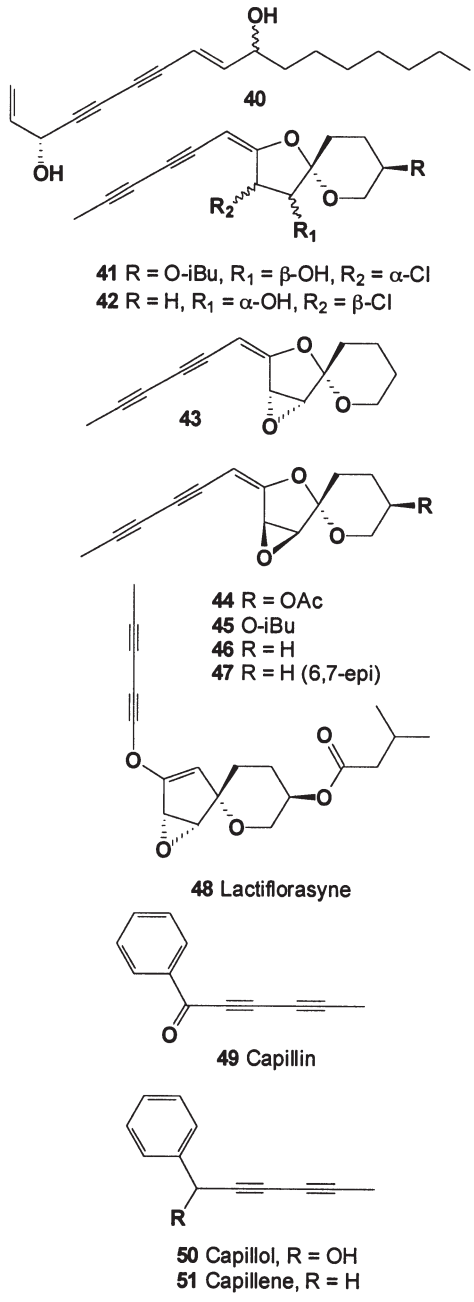


TABLE 2
Inhibitory Effects of Some Polyacetylenes on TPA-Induced O_2^- Generation[a] (ref. 78)

Compounds	10 (μM)	50 (μM)	100 (μM)	IC_{50} (μM)
41	29	67	75	28.0
42	19	47	60	56.0
44	32	61	80	29.0
45	60	>99	NT	7.6
46	15	51	77	47.0
47	NT	55	78	43.0

[a]The maximal SD for each experiment was 5% from at least duplicate tests. NT, not tested; TPA, 12-*O*-tetradecanoylphorbol-13-acetate. IC_{50}, concentration that is inhibitory to 50% of test subjects.



promotion in ICR mouse skin were reported (81). AL-1 (**45**) strongly inhibited tumor promoter-induced Epstein-Barr virus activation in Raji cells (IC_{50} 0.5 μM), the effect being comparable with or even stronger than that of curcumin, a well-known antioxidative chemopreventive from turmeric. The cytotoxic compound lactiflorasyne (**48**) containing a spiro system has been isolated from the flowers and leaves of *A. lactiflora* (81–83).

Capillin (**49**) was isolated from *Artemisia capillaris* (fragrant wormwood), and its inhibitory activity on carcinogenic Epstein-Barr virus demonstrated (84-86). Capillin, capillol (**50**), and capillene (**51**) were obtained from extracts of *A. sco-*

TABLE 3
Cytotoxicity[a] of Gummiferol (54) Isolated from *Adenia gummifera* (ref. 103)

Cell line	ED_{50} (μg/mL)	Cell line	ED_{50} (μg/mL)
BCA-1	0.2	KB-V+	0.3
HT-1080	0.1	P388	0.03
LUC-1	0.9	A-431	0.5
MEL-2	1.3	LNCaP	0.2
COL-1	0.6	ZR-75-1	0.2
KB	0.3	U-373	0.05
KB-V-	0.4		

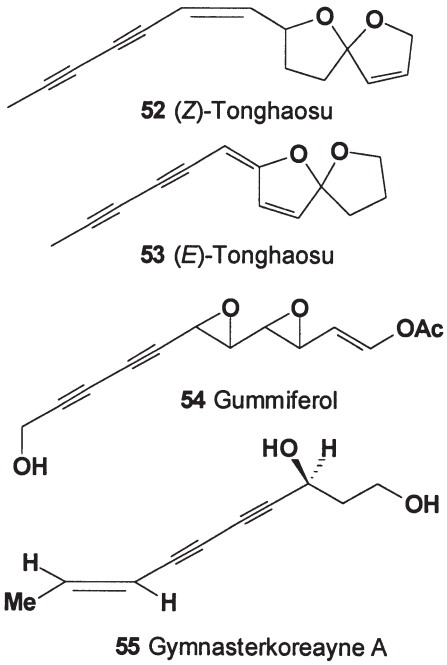
[a]Key to cell lines used: BC1, human breast cancer; HT-1080, human fibrosarcoma; LUC-1, human lung cancer; MEL-2, human melanoma; COL-1, human colon cancer; KB, human oral epidermoid carcinoma; KB-V+, multidrug-resistant KB assessed in the presence of vinblastine (1 μg/mL); KB-V–, multidrug-resistant KB assessed in the absence of vinblastine; P388, murine lymphoid neoplasm; A-431, human epidermoid carcinoma; LNCaP, hormone-dependent human prostate cancer; ZR-75-1, hormone-dependent human breast cancer; U-373, human glioblastoma. ED_{50}, dose that is effective in 50% of test subjects

paria (redstem wormwood) (87,88), from aerial parts of *A. variabilis* (89), and from the essential oil of *Santolina rosmarinifolia* (Holy flax, Compositae) (90). Concentrations (1–10 μM) of capillin (**49**) from *A. monosperma* inhibited cell proliferation of four human tumor cell lines—colon carcinoma HT29, pancreatic carcinoma MIA PaCa-2, epidermoid carcinoma of the larynx HEp-2, and lung carcinoma A549—by inducing DNA fragmentation and cell death (91). The oil content increased in the aerial parts of *A. scoparia* from the rosette stage to budding (from 0.21 to 2.5%) and flowering (0.23 to 2.6%). Concomitantly the capillene (**51**) content increased from 42.4 to 79.8% and that of capillin (**49**) decreased from 45.0 to 4.4% (92). Capillene and capillin were the main components in both the roots and leaves of *A. capillaris* growing at riverside sites (93).

German chamomile (*Matricaria recutita,* Asteraceae) oils containing ≥1% by wt of natural *cis*- and *trans*-spiro ether polyynes were obtained by steam distillation of fresh chamomile. Isolated compounds were named as *Z*-tonghaosu (**52**) and *E*-tonghaosu (**53**) (94–96), and they showed inhibiting activity on human lung cancer cell line as well as insecticidal and considerable antifeedant properties (97–102). A new polyacetylenic diepoxide compound, gummiferol (**54**), was isolated from the leaves of *Adenia gummifera* (Passifloraceae) by KB (carcinoma cells) cytotoxicity-guided fractionation. It exhibited cytotoxic actions on KB cell lines and a broad cytotoxic spectrum against 10 other human cancer cell lines (Table 3) (103). *Adenia gummifera* is used to improve animal health in Tanzania.

Polyacetylenic compounds isolated from roots of *Gymnaster koraiensis* (Compositae) included the gymnasterkoreaynes A to F (compounds **55–62**, respectively) as well as 1,9,16-heptadecatrien-4,6-diyn-3,8-diol (**59**) and 1,9,16-heptadecatrien-4,6-diyn-8-ol (**60**), and were separated by bioassay-guided fractionation using the L-1210 tumor cell line as a model for cytotoxicity (104).

Gymnasterkoreaynes A–F are linear diacetylenes and are



structurally related to falcarinol, panaxynol, panaxydiol, and panaxytriol. In isolated form, gymnasterkoreaynes B (**56**), C (**58**), F (**62**), and 1,9,16-heptadecatrien-4,6-diyn-8-ol (**60**) exhibited significant cytotoxicity against L-1210 tumor cells with ED_{50} values of 0.12–3.3 μg/mL (Table 4). In addition, gymnasterkoreaynes A–F showed considerable antiproliferative activity against various cancer cells, inhibition of NO production, and inhibition of acyl-CoA:cholesterol acyltransferase (105).

The antitumor acetylenic compounds 2-*cis*-dehydro-matricaria acid (**63**), 2-*trans*-dehydromatricaria acid (**64**), *cis*-dehy-

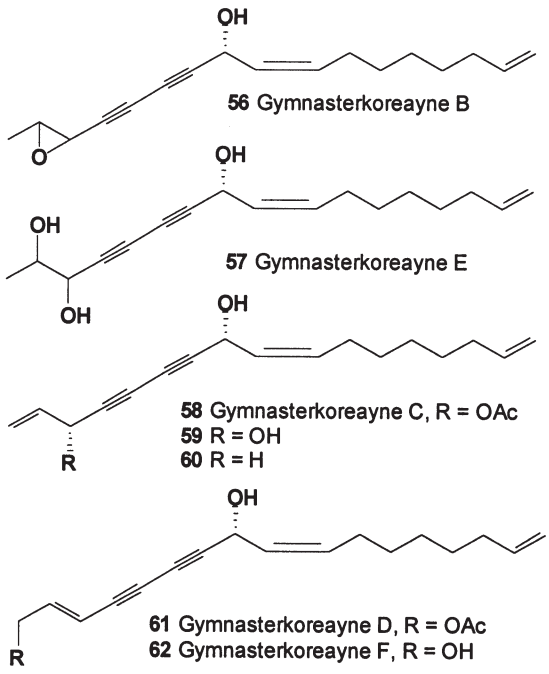


TABLE 4
Cytotoxicity[a] of the Acetylenic Compounds Isolated from *Gymnaster koraiensis* Against L-1210 Tumor Cell Line (ref. 104)

Compound	ED_{50} (μg/mL)	Compound	ED_{50} (μg/mL)
55	>10	**59**	10.4
56	3.3	**60**	0.12
57	9.6	**61**	7.7
58	2.1	**62**	3.1

[a]For abbreviation see Table 3.

dromatricaria Me ester (**65**), and *trans*-dehydromatricaria Me ester (**66**) were obtained from roots of *Solidago virga-aurea* (Compositae) (106,107), and also from other species (108–111).

Polyacetylene (–)-17-hydroxy-9,11,13,15-octadecatetraynoic acid (**67**), referred to as minquartynoic acid, from *Minquartia guianensis* stem bark showed cytotoxic activity against P388 murine lymphocytic leukemia *in vitro*. The P388 ED_{50} of the pure compound was 0.18 μg/mL, and it was also active in the brine shrimp larvicidal bioassay with an LC_{50} of 5.06 μg/mL (95% confidence interval 3.68–6.98 μg/mL). These biological activities could account for the alleged efficacy of the plant in folk usage (112,113).

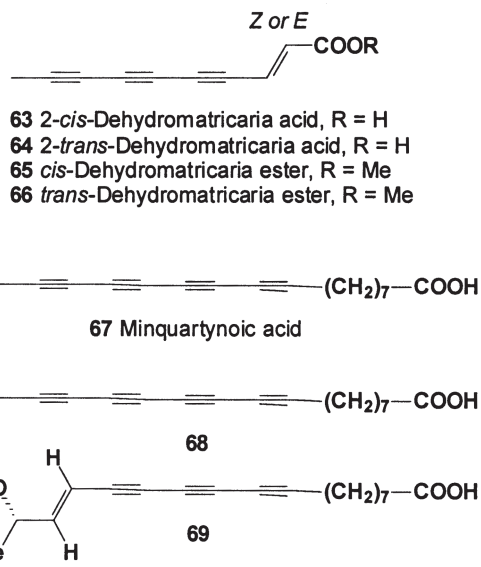
More recently, minquartynoic acid was isolated from the air-dried bark of *Coula edulis* (114), the twigs of *Ochanostachys amentacea* (both plants belonging to the Olacaceae) (115,116), and from *Minquartia guianensis* bark (117). In addition, acetylenic acids **68** and **69** were isolated from the twigs of *O. amentacea* (115,116), and their cytotoxic activities determined (Table 5). Minquartynoic acid (**67**) also showed moderate *in vitro* activity against *Plasmodium falciparum* and *Leishmania major* and strongly inhibited phytohemagglutinin A-induced proliferation of human lymphocytes (118).

A dichloromethane extract of celery root (*Apium graveolens*) yielded falcarinol (**6**), falcarindiol (**21**), panaxydiol (**70**), and 8-OMe-falcarindiol (**71**) (119). Its nonpolar extracts as

TABLE 5
Cytotoxic Activity[a] for Compounds 67–69

Cell lines[b]	67	68	69
BC1	3.5	>20	>20
Lu1	4.1	14.6	>20
Col2	5.5	>20	9.9
KB	3.7	2.6	1.1
KB-V+	2.8	11.9	>20
KB-V–	4.3	13.4	>20
LNCaP	1.6	9.2	0.30
SW626	4.1	19.4	0.36
SKNSH	1.4	6.7	3.7
M109	3.7	10.1	5.4

[a]ED_{50}, μg/mL. From Reference 115. For abbreviation see Table 3.
[b]Key to cell lines used: BC1, human breast cancer; Lu1, human lung cancer; Col2, human colon cancer; KB, human oral epidermoid carcinoma; KB-V+, multidrug-resistant KB assessed in the presence of vinblastine (1 μg/mL); KB-V–, multidrug-resistant KB assessed in the absence of vinblastine; LNCaP, hormone-dependent human prostate cancer; SW626, human ovarian cancer; SKNSH, human neuroblastoma cancer; M109, mouse lung cancer.



well as those obtained from roots and bulbs of carrots, fennel, parsley, and parsnip were separated by HPLC. All species contained polyacetylenes, although carrots and fennel only in minor amounts. In addition, by using the annexin V-PI assay, the cytotoxicity of the compounds **6**, **21**, **70**, and **71** was evaluated. Falcarinol proved to be the most active, with a pronounced toxicity against an acute lymphoblastic leukemia cell line CEM-C7H2 (IC_{50} = 3.5 mg/mL).

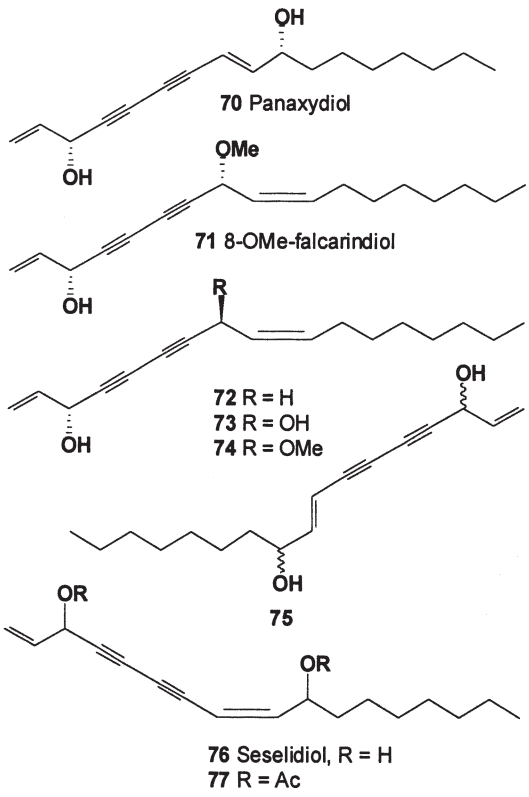
Polyacetylenes (**72–75**) from *Apium graveolens* showed medium-level cytotoxicity against certain leukemia, lymphoma, and myeloma cell lines with IC_{50} values of approximately 30 μM (Table 6). Only falcarinol showed much higher activity against one cell line, CEM-C7H2, with an IC_{50} value of 3.50 μM. Colorectal carcinoma cell lines were less sensitive to polyacetylenes in general, with IC_{50} values above 100 μM for compound **73** against both cell lines and for compound **74** against HT2912 cells. However, compound **74** also displayed medium-level activity against HRT-18 cells, and compounds **72** and **75** displayed medium-level activities against both cell lines (IC_{50} values between 30 and 64 μM).

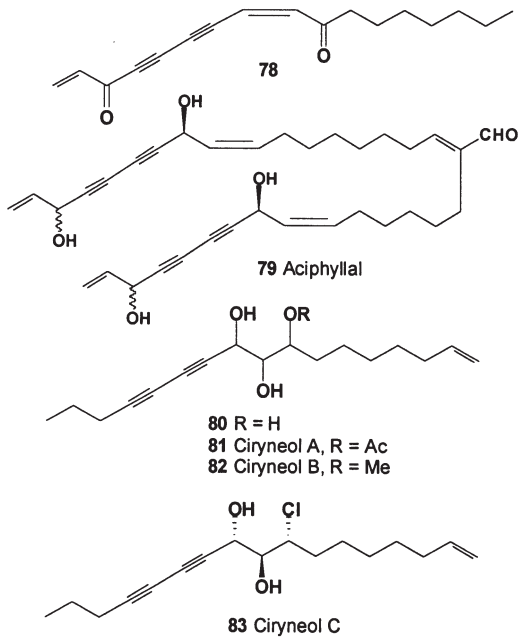
Seselidiol (**76**) has been isolated from the roots of *Seseli mairei* (120). Seselidiol, its acetate (**77**), and compound **78** showed moderate cytotoxicity against KB, P388, and L-1210 tumor cells (Table 7).

TABLE 6
Cytotoxicity[a] of Polyacetylenes Isolated from *Apium graveolens*

Compound	A	B	C	D	E
72	3.5	30.1	27.4	42.3	63.9
73	29.8	31.8	29.6	>100	>100
74	31.8	32.1	29.3	34.7	>100
75	29.2	29.5	27.8	29.8	32.3

[a]IC_{50}, μg/mL (ref. 119). Abbreviations: A, CEM-C7H2 (human lymphoblastic leukemia); B, U937 (human monocytic lymphoma); C, RPMI (human nasal epithelial cells); D, HRT-18 (adenocarcinoma); E, HT2912 (human brain neoplasm). For other abbreviation see Table 2.





The novel dimeric C_{34}-polyacetylene aciphyllal (**79**) was isolated from the New Zealand subalpine herb (giant speargrass) *Aciphylla scott-thomsonii* (Apiaceae, tribe Apieae) by Perry and co-workers (121) and synthesized by Patnam and coworkers (122). This was the first report on very long chain polyacetylenes (up to C_{45}) isolated from marine sponges. Aciphyllal showed weak cytotoxic activity against P388 cells (IC_{50} > 25 μg/mL) and antifungal activity against *Trichophyton mentagrophytes*.

The polyacetylene compounds heptadeca-1-ene-l,13-diyne-8,9,10-trio1 (**80**) and ciryneols A–C (**81–83**) were isolated from *Cirsium japonicum* (Compositae). These compounds inhibit the growth of KB cells *in vitro* (123–125). Ciryneol C (**83**) was also isolated from *C. rhinoceros* (126). *Scurrula atropurpurea* (Loranthaceae), a parasitic plant that attacks the tea plant *Thea sinensis* (Theaceae), is called "benalu teh" in Indonesia, and the whole plant (stems and leaves) has been traditionally used for the treatment of cancer in Java (Indonesia). Three acetylenic acids—octadeca-8,10-diynoic (**84**), (*Z*)-octadec-12-ene-8,10-diynoic (**85**), and octadeca-8,10,12-triynoic (**86**) acids—were isolated from aqueous acetone extracts of stems and leaves. All compounds showed inhibitory activity against cancer cell invasion (MM1) *in vitro* (Table 8) (127,128). Five C_{16}-acetylenic FA—hexadec-8-ynoic (**87**), hexadec-10-ynoic (**88**), hexadeca-8,10-diynoic (**89**), hexadeca-6,8,10-triynoic (**90**), and hexadeca-8,10,12-triynoic (**91**) acids—were prepared, and their inhibitory activity against cancer cell invasion was examined (129). The results indicated that the natural C_{18}-triyne FA (**86**) and the synthetic C_{16}-triyne FA (**90** and **91**) inhibit cancer cell invasion in spite of their simple chemical structures.

Bioassay-directed fractionation of the dried roots of *Asparagus cochinchinensis* (Asparagaceae) led to the isolation of some active compounds (130–132), including a new acetylenic

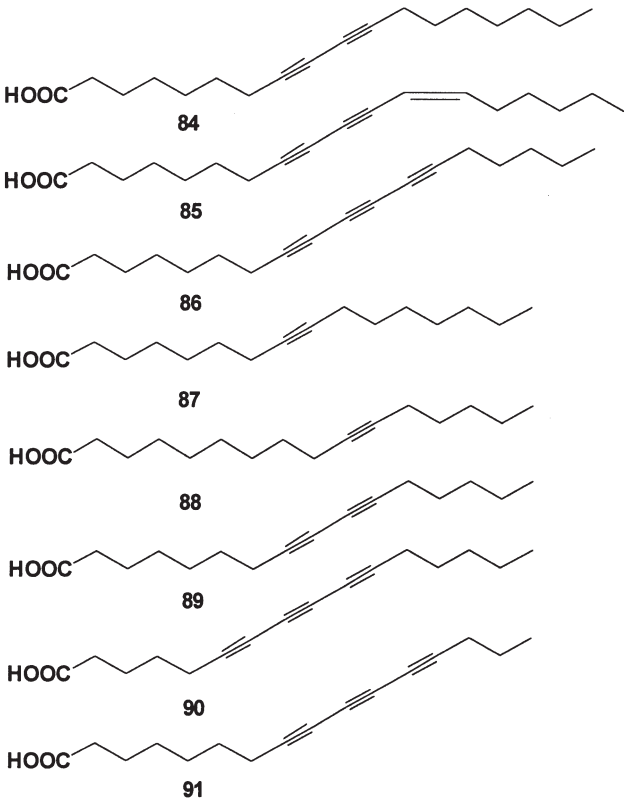
TABLE 7
Cytotoxicity[a] of Acetylenic Metabolites Isolated from *Seseli mairei*

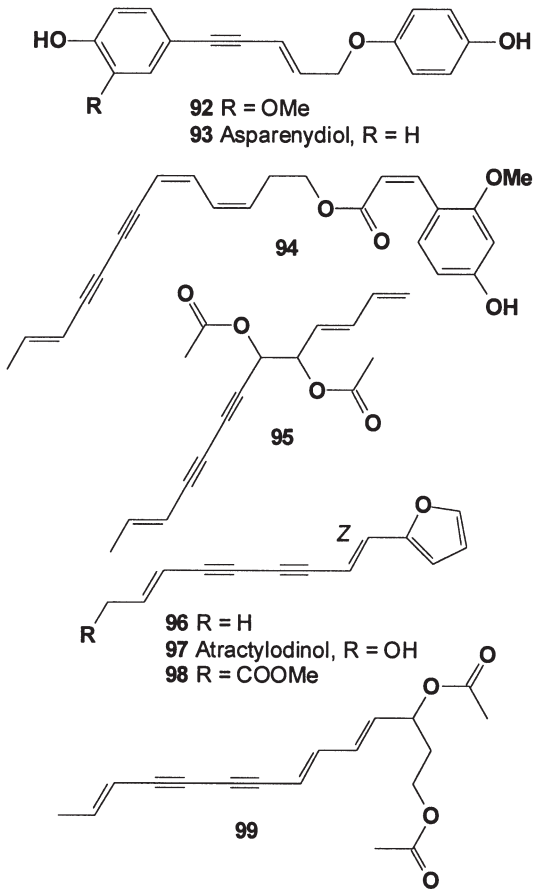
Compound	KB	HCT-8	P388	L-1210
76	1.0	10.0	4.9	3.3
77	4.0	7.8	5.2	5.5
78	>10	3.7	>10	>10

[a]ED_{50}, μg/mL (ref. 120). Key to cell lines used: KB, human oral epidermoid carcinoma; HCT-8 = human colon carcinoma; P388, murine lymphoid neoplasm; L-1210, murine leukemic cells. For abbreviation see Table 3.

TABLE 8
Inhibitory Activities of C_{16}- and C_{18}-Acetylenic Acids on Cancer Cell Invasion (MM1; ref. 127)

Compound	Concentration (μg/mL)	Inhibitory activity (%)
84 Natural	10	61.1
85 Natural	10	89.8
86 Natural	10	99.4
86 Natural	5	94.9
86 Natural	2.5	45.6
87 Synthetic	10	82.4
88 Synthetic	10	77.2
89 Synthetic	10	85.6
90 Synthetic	10	95.7
90 Synthetic	5	85.4
90 Synthetic	2.5	50.3
91 Synthetic	10	98.7
91 Synthetic	5	90.7
91 Synthetic	2.5	60.5





derivative, 3″-methoxyasparenydiol (**92**), and asparenydiol (**93**). These compounds demonstrated moderate cytotoxicities in a panel comprised of KB, Col-2, LNCaP, Lu-1, and human umbilical vein endothelial cells (HUVEC) cells, with IC_{50} values ranging from 4 to 20 μg/mL (Table 9). The dried roots of *A. cochinchinensis* are used in Laos to treat chronic fever (Lao name of plant: Kheua Ya Nang Xang). The plant has a long history of use for treating fever, cough, kidney diseases, and benign breast tumors in China.

From the rhizomes of *Atractylodes lancea* were isolated bioactive compounds **94–99** (133–136). Only **94** showed strong inhibition of 5-LOX and COX-1 activity [IC_{50} (5-LOX) = 3.4 μM; IC_{50} (COX-1) = 1.1 μM]. The activity of the other compounds is shown in Table 10. Crude extracts of seven species of the genus *Argyranthemum* were tested for potential cytotoxic and antimicrobial activities (137,138). *Argyranthemum adauctum, A. foeniculaceum*, and *A. frutescens* were effective against both Gram-positive and Gram-negative bacteria and showed cytotoxic activity against HeLa and Hep-2 cell lines. Cytotoxic activity of **100–110** against HeLa and Hep-2 cells is shown in Table 11.

Some identified compounds from *Angelica* species showed antimicrobial, anticancer, antitumor, analgesic, anti-inflammatory, hepatoprotective, and nephroprotective activities. The $CHCl_3$ extract from *A. japonica* root showed high inhibitory activity on human gastric adenocarcinoma (MK-1) cell growth. From this extract, a new furanocoumarin named japoangelone and four furanocoumarin ethers of falcarindiol, named japoangelols A (**111**, ED_{50}, 8.5 μg/mL), B (**112**, ED_{50}, 7.2 μg/mL), C

TABLE 9
Cytotoxic Activities[a] of Compounds Isolated from *Asparagus cochinchinensis*

Compound	A	B	C	D	E	F
92	12.0	>20	>20	19.7	>20	<5.0
93	2.4	>20	>20	19.8	>20	<5.0

[a]IC_{50}, μg/mL. From Reference 130. Abbreviation: A, KB (human oral epidermoid carcinoma); B, COL-2 (human colon cancer); C, LNCaP (hormone-dependent human prostate cancer); D, LuI (left upper-lobe lung cancer); E, HUVEC (human umbilical vein endothelial cells); F, HOG.R5 (green fluorescent protein-based reporter cell line). For other abbreviation see Table 2.

TABLE 10
***In vitro* Inhibitory Actions[a] of Compounds Isolated from *Atractylodes lancea* on Leukotriene (5-LOX) and Prostaglandin (COX-1) Biosyntheses**

Compound	IC_{50} (5-LOX)	IC_{50} (COX-1)
94	3.4	1.1
95	46.3	>200
96	158.3	176.6
97	>200	>200
98	>200	>200
99	27.9	20.5

[a]IC50, μM. COX, cyclo-oxygenase; for other abbreviation see Table 2. From Reference 133.

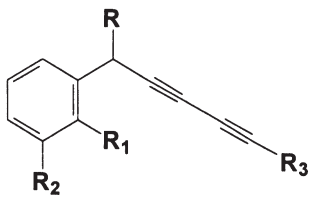
TABLE 11
Cytotoxic Activity[a] of Acetylenic Compounds Isolated from *Argyranthemum frutescens*

Compound	HeLa (IC_{50}, μg/mL)	Hep-2 (IC_{50}, μg/mL)
100	<50 inactive	<50 inactive
101	37.0	>50
102	30.0	29.0
103	48.0	>50
104	18.0	26.0
105	<50 inactive	<50 inactive
106	<50 inactive	<50 inactive
107	<50 inactive	<50 inactive
108	<50 inactive	<50 inactive
109	44.0	>50
110	<50 inactive	<50 inactive

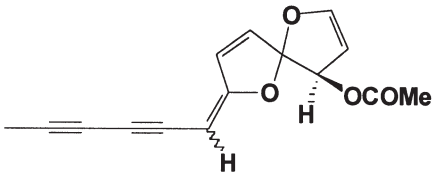
[a]From Reference 138. Key to cell lines used: HeLa, human carcinoma of cervix; Hep-2, human carcinoma of larynx. For abbreviation see Table 2.

(**113**, ED_{50}, 7.4 μg/mL), and D (**114**, ED_{50}, 8.4 μg/mL) were isolated and tested. In addition, three other polyacetylenes were isolated and tested in the MTT assay: panaxynol (ED_{50}, 0.3 μg/mL), falcarindiol (**21**) (ED_{50}, 3.2 μg/mL), and 8-OMe-falcarindiol **71** (ED_{50}, 3.2 μg/mL). Moderate activity of compounds **111**–**114** against HeLa and B16F10 cell lines were also reported (139,140).

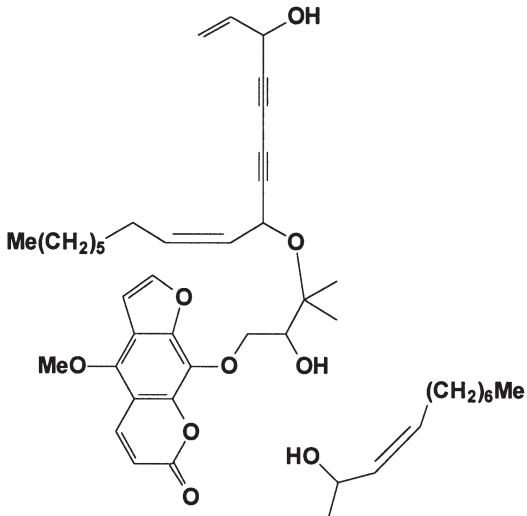
A few acetylenic phytosterols have been found. The first acetylenic sterols (**115–117**) from *Gymnostemma pentaphyl-*



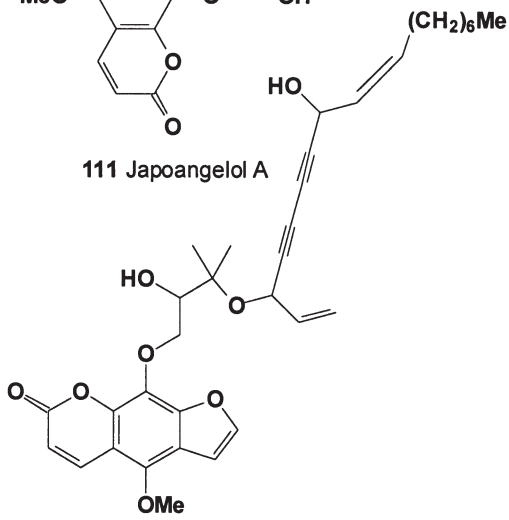
100 R = $OCOOCH_2CH(Me)_2$, R_1 = COOMe, R_2 = OMe, R_3 = Me
101 R = $OCOOCH2CH(Me)_2$, R_1 = COOMe, R_2 = OMe, R_3 = Me
102 R = OAc, R_1 = COOMe, R_2 = OH, R_3 = Me
103 R = OAc, R_1 = COOMe, R_2 = OMe, R_3 = Me
104 R = OAc, R_1 = COOMe, R_2 = H, R_3 = Me
105 R = OAc, R_1 = R_2 = H, R_3 = Me
106 R = H, R_1 = COOMe, R_2 = OMe, R_3 = Me
107 R = (=O), R_1 = COOMe, R_2 = OMe, R_3 = Me
108 R = OAc, R_1 = COOMe, R_2 = OMe, R_3 = H



109 H, 6*E*
110 H, 6*Z*



111 Japoangelol A



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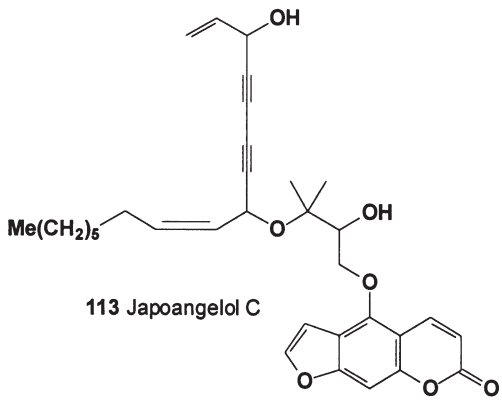

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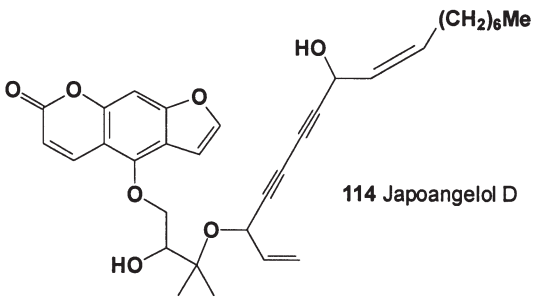
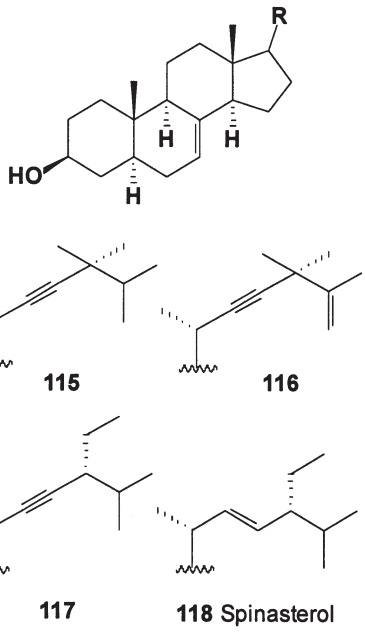
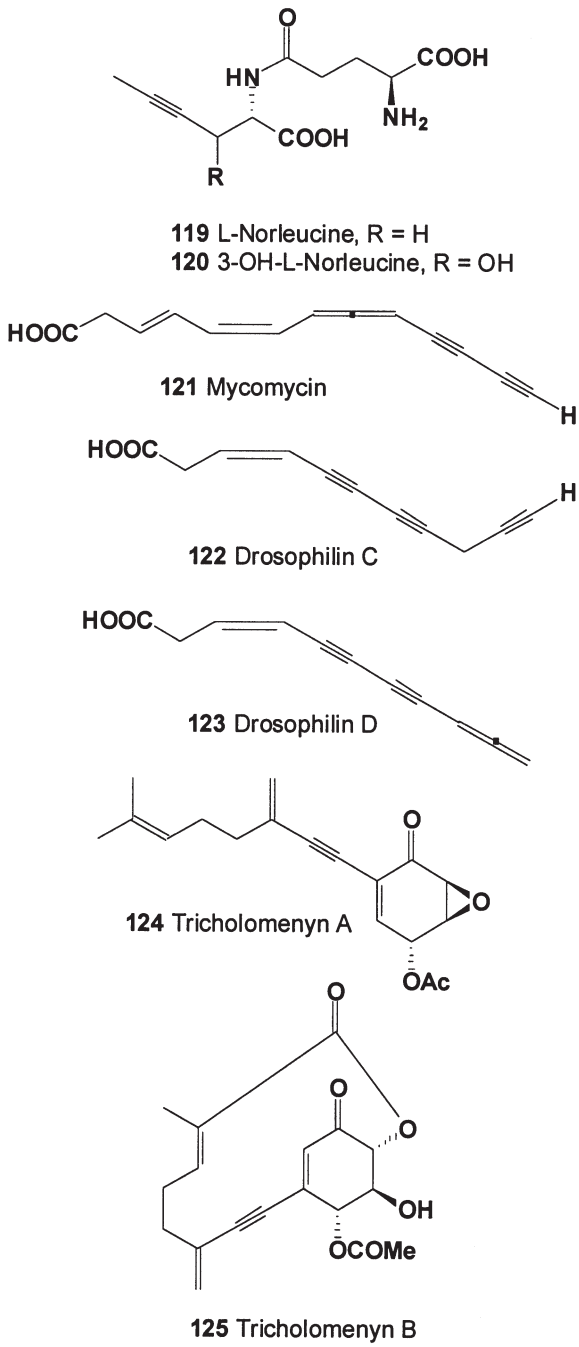


114 Japoangelol D





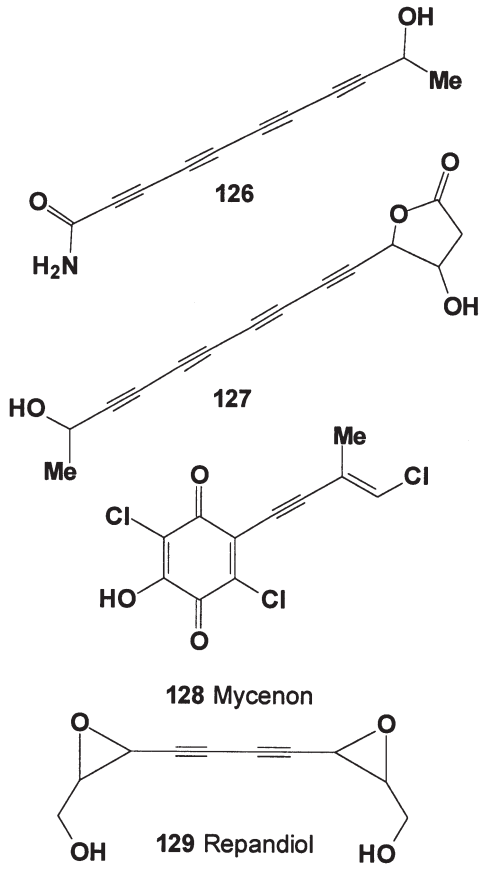
lum (Cucurbitaceae) were isolated by Akihisa and co-workers in 1989 (141).

Acetylenic sterols having a same structure (except for the side chains) as the anticancer agent spinasterol (**118**) were isolated from *Pueraria* roots (*P. mirifica* from Thailand and *P. lobata* from Korea) (142). These plants are used as a rejuvenating folk medicine in Thailand and China. The ethanol extracts had significant antiproliferative effects on breast cancer cell lines, including MCF-7, ZR-75-1, MDA-MB-231, SK-BR-3, and Hs578T. Spinasterol inhibited the growth of some breast cancer cell lines (MCF-7, MDA-MB-231) in a dose- and time-dependent manner, as well as the growth of ovarian (2774) and cervical cancer cells (HeLa) (142). The occurrence of spinasterol has also been reported in *Cucurbita moschata* (143), *Conyza blinii* (144), *Gypsophila oldhamiana* (145), *Gordonia ceylanica* (146), and *Acacia cedilloi* (147). The anticancer activity of spinasterol was demonstrated *in vivo* in studies that showed that it greatly decreased the incidence of skin tumors without co-carcinogen or co-tumor promoter activities (148).

FUNGAL ACETYLENIC METABOLITES

Fungal species produce many different acetylenic metabolites, but only few of them show cytotoxic, antitumor, and/or related activities (149–154). Two glutamyl peptides, γ-glutamyl-L-2-aminohex-4-ynoic acid (**119**) and γ-L-glutamyl-L-erythro-2-amino-3-hydroxyhex-4-ynoic acid (**120**), were isolated from the fruit bodies of *Tricholomopsis rutilans* (155–157). Derivatives of these amino acids showed antiviral, anticholesterol, and anticancer activities (158–166).

Mycomycin (**121**), isolated in 1950 by Jenkins (167), is used not only as a therapeutic agent for tuberculosis (168–170) but also for treatment of late-stage inoperable primary hepatocellular carcinoma (171,172). More recently, antibiotic 07F275 [also known as mycomycin (**121**)], which belongs to the allenic polyacetylene family, has been produced by submerged fermentations of fungal culture LL-07F275 (173). Acetylenic metabolites named drosophilins C (**122**) and D (**123**) have been isolated from fungal culture of *Drosophila subatrata* (now classified as *Psathyrella subatrata*) (174–176). These compounds showed antibacterial, antimicrobial, and antifungal activities, and they inhibited bacteriophage growth (174–180). Tricholomenyns A (**124**) and B (**125**) were isolated from the fruiting bodies of *Tricholoma acerbum* (181,182). The tricholomenyns efficiently inhibit mitosis of T-lymphocyte cultures and are potent as anticancer agents.



Two bioactive polyynes, 10-hydroxyundeca-2,4,6,8-tetraynamide (**126**) and 3,4,13-trihydroxy-tetradeca-5,7,9,11-tetraynoic acid-γ-lactone (**127**), were isolated from cultures of the fungus *Mycena viridimarginata* (183,184). Compound **126** was highly active against Gram-positive and Gram-negative bacteria, yeasts, filamentous fungi, and Ehrlich ascites carcinoma. Compound **127** had similar though less pronounced biological activities. A novel chlorinated benzoquinone antibiotic, mycenon (**128**), was isolated from the culture broth of a basidiomycete, *Mycena* sp., and shown to inhibit isocitrate lyase (EC 4.1.3.1) (185).

Repandiol (**129**), a new cytotoxic diepoxide [(2*R*,3*R*,8*R*, 9*R*)-4,6-decadiyne-2,3:8,9-diepoxy-1,10-diol], was isolated from the mushrooms *Hydnum repandum* and *H. repandum* var. *album* (186–188). Repandiol displayed pronounced cytotoxic effects against various tumor cells. It was found to form interstrand cross-links of DNA, linking deoxyguanosines on opposite strands primarily within the 5′-GNC and 5′-GNNC sequences preferred by diepoxyoctane. However, repandiol was a significantly less efficient cross-linker than diepoxy-alkanes (diepoxyoctane and diepoxybutane) (189).

BACTERIAL AND CYANOBACTERIAL ACETYLENIC METABOLITES

Microorganisms produce a large variety of biologically active substances representing a vast diversity of fascinating molecular architecture not available in any other living systems. During the last two decades, quite a few active compounds having an enediyne unit have been isolated and identified from soil bacteria and identified. In 1987, Lederle (190–193) and Bristol-Myers (194,195) groups revealed unprecedented molecular architecture of a new class of natural products, the so-called enediyne anticancer antibiotics. The enediynes are characterized by the presence of an unsaturated core with two acetylenic groups conjugated to a double bond or to an incipient double bond. They are categorized into two subfamilies possessing either 9-membered ring chromophore cores or 10-membered rings.

Calicheamicin γ_1 (**130**), the most prominent member of the calicheamicins, was isolated from *Micromonospora echinospora* ssp. *calichensis* and is a remarkable piece of engineering by Nature, which has perfectly constructed the molecule to endow it with its extraordinary chemical and biological properties (196). One can mention, in particular, its efficiency in the biochemical prophage induction assay at concentrations <1 pM, high antibacterial activity, and extreme potency against murine tumors such as P388 and L-1210 leukemias and solid neoplasms such as colon 26 and B16 melanoma with optimal doses of 0.15–5.0 μg/kg.

The esperamicins represent a class of antitumor antibiotics revealing extremely high cytotoxicity. A family of potent esperamicins showing a broad spectrum of antimicrobial and antitumor activities in murine systems has been identified in cultures of *Actinomadura verrucosospora* (193,194). The esperamicins are characterized by the presence of a particular central core. Unusual features of the core include a bicyclo[7.3.1] ring system, an allylic trisulfide attached to the bridging atom, a 1,5-diyn-3-ene as part of the ring system, and an α-3-unsaturated ketone in which the double bond is at the bridgehead of the bicyclic system. Esperamicin A_1 (**131**) contains four sugars attached to the bicyclic core and an aromatic chromophore attached to one of the sugars. Cytotoxicities of esperamins A_1 (**131**), C (**133**), and D (**134**) are presented in Table 12. Another compound, esperamicin P (**132**), isolated from a fermentation broth of *A. verrucosospora* was found to differ from esperamicin A_1 by possessing a methyl tetrasulfide moiety instead of a methyl trisulfide (197). It was active against xenografted tumors in mice and exhibited antimicrobial activity. It also showed antitumor activity against cultured cell lines with IC_{50} values (in μg/mL) of 0.01 against A549 human lung and A549 (VP-resistant) cells; 0.02 against B16-PRIM murine

TABLE 12
Cytotoxicity[a] of Esperamicins

Compound	A	B	C	D	E
131	0.0045	0.0003	0.0018	0.0083	0.0022
133	0.85	0.067	0.71	1.5	1.04
134	4.6	0.87	2.7	>12	4.9

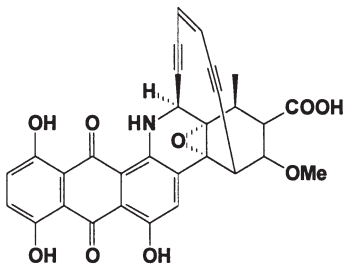
[a]IC_{50}, μg/mL. From Reference 191. Abbreviations: A, B16-F10 (murine melanoma); B, HCT116 (human colon carcinoma); C, H2981 (human lung carcinoma); D, MOSER (adenocarcinoma); E, SW900 (human lung neoplasm).

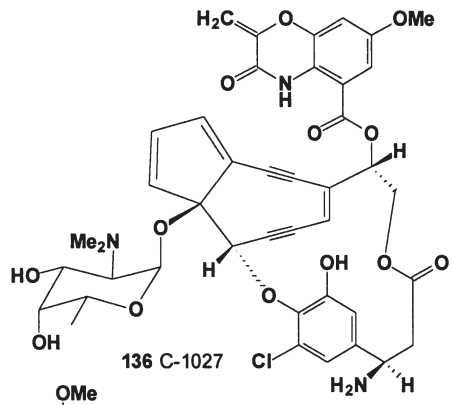
melanoma cells, and <0.002 against HCT-116 human colon and HCTNP35 (VP-resistant human colon) cells (197).

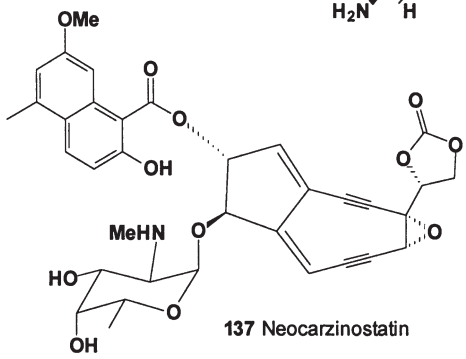
Dynemicin A (**135**), a new antibiotic with 1,5-diyn-3-ene and anthraquinone subunits, was isolated from the culture broth of *Micromonospora chersina* sp. nov. M956-1 (198). It was especially active against Gram-positive bacteria, prolonged the life span of mice inoculated with P388 leukemia, and displayed significant activity against P388 leukemia and 316 melanoma in mice.

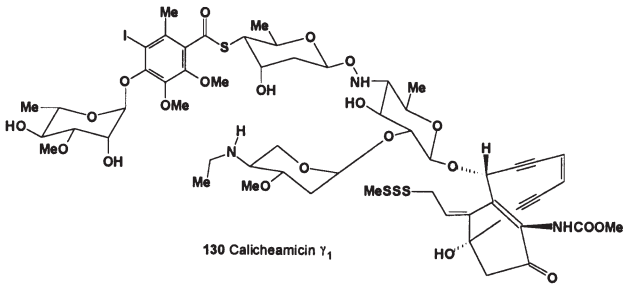
Antitumor antibiotic C-1027 (**136**) isolated from *Streptomyces globisporus* (199–202) showed pronounced cytotoxicity against KB carcinoma cells (IC_{50} 0.1 ng/mL) (203) and was effective as an antitumor drug in experiments on tumor-bearing mice (204–206). The metabolites, belonging to a steadily growing enediyne family, now comprise nine natural products: neocarzinostatin (**137**), which was isolated from *S. carzinostaticus* (207–213), kedarcidin (**138**) from *Actinomycete* L585-6 (214–218), maduropeptin (**139**) from *Actinomadura madurea* (219,220), and N1999A2 (**140**) from *Streptomyces* sp. AJ9493 (221–223). The representatives of this group are probably the most potent anticancer agents discovered. Some of them are 5–8000 times more effective than adriamycin, an antibiotic widely used for antitumor treatment (224,225).

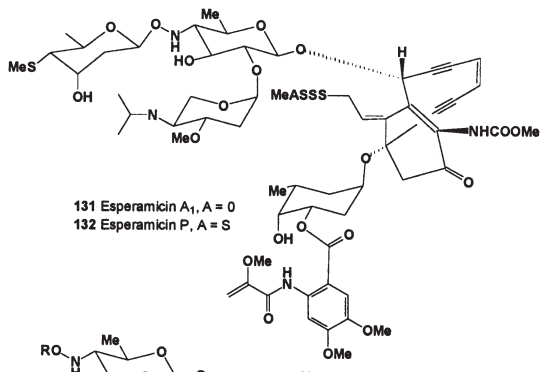
Neocarzinostatin (**137**) showed antibacterial activity (LD_{50}, μg/mL) against Gram-positive organisms: *Bacillus subtilis*, 32;

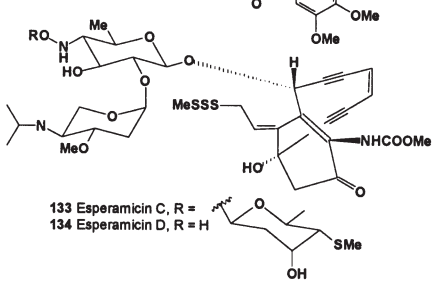






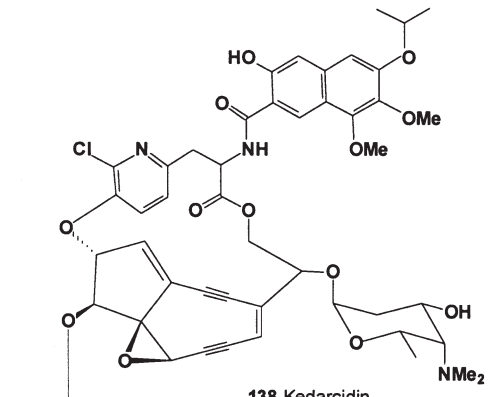




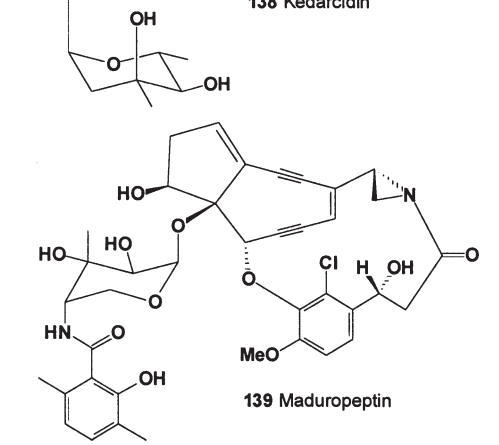


Staphylococcus aureus, 16; *S. aureus*, 32; *S. aureus*, 8; *S. aureus*, 16; *Sarcina lutea*, 2; and *S. lutea*, 2. In mice with ascitic sarcoma 180, 3.2 mg/kg daily was tolerable. The LD_{50} was 30 mg/kg. In doses of 0.1–3.2 mg/kg/d, neocarzinostatin inhibited tumor growth with a therapeutic index (also known as the therapeutic ratio or margin of safety) of 32; ranges of 0.8–3.2 μg/kg/d gave 100% survival. Doses of 0.2–3.2 mg/kg/d in leukemia SN-36-bearing mice significantly prolonged survival of animals.

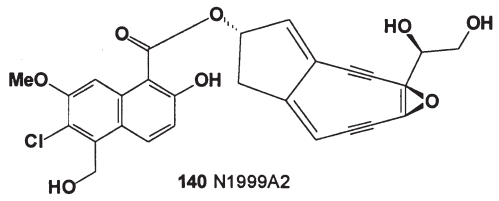
Kedarcidin (**138**) showed antitumor activity against implanted P388 leukemia (3.3 μg/mL/kg), and B16 melanoma (2 μg/kg) in mice. It was also effective against Gram-positive, but not against Gram-negative, bacteria (226,227). The antibiotic maduropeptin (**139**) was active against Gram-positive bacteria and highly cytotoxic to tumor cells. It produced significant prolongation of survival of mice implanted with P388 leukemia and B16 melanoma (228). Antibiotic N1999A2 (**140**) strongly inhibited growth of various tumor cell lines (with IC_{50} values varying from 10^{-12} to 10^{-8} M) and bacteria (229). For details on the chemistry and biological activities of this family of antibiotics see recent publications (230–233).



138 Kedarcidin



139 Maduropeptin



140 N1999A2

Cyanobacteria include nearly 2,000 species, growing as single cells, filaments of cells, or through various colonial associations (234–239). This indicates a high degree of biological adaptation, which has enabled these organisms to thrive and compete effectively in nature. Many of them produce toxic secondary metabolites, in particular nerve and liver toxins, as a form of defense against herbivores. At the same time, some cyanobacterial species represent a source of interesting active metabolites, including acetylenic compounds that possess selective cytotoxicities and that may prove useful for development into commercial drugs (240–246). The ubiquitous tropical cyanobacterium *Lyngbya majuscula* is a prolific producer of bioactive metabolites: Approximately 30% of all natural products reported from marine cyanobacteria have been isolated from this species. The plethora of structurally diverse secondary metabolites isolated from *L. majuscula* exhibits a variety of bioactivities including antifeedant, molluscicidal, antiproliferative, and immunosuppressive properties. More than half of the known secondary metabolites of the species are either cyclic or linear lipopeptides, some of them having an acetylenic unit.

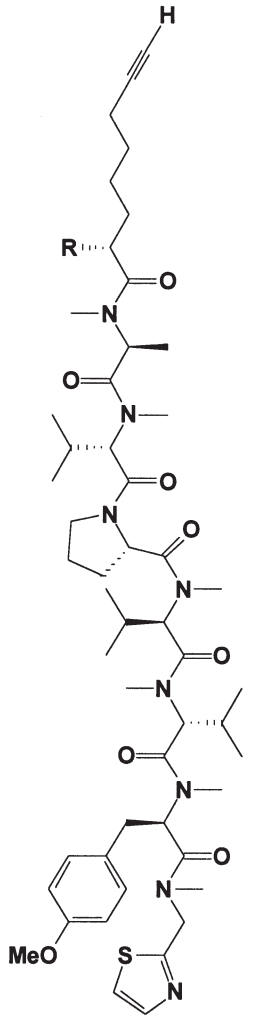
The linear lipopeptides named apramides A (**141**), B (**142**), and G (**143**) have been isolated from the cytotoxic fraction of *L. majuscula* collected at Apra Harbor (Guam). Apramide G showed cytotoxic activity, with IC_{50} values of 33 and 11 ng/mL against KB and LoVo cells, respectively (247,248). Four new metabolites have been isolated from *L. majuscula* collected at Boca del Drago Beach, Bocas del Toro, Panama. These compounds were assigned the trivial names dragonamide (**144**), pseudodysidenin, dysidenamide, and nordysidenin. Dragonamide exhibited cytotoxic activity against P388, A-549, HT-29, and MEL-28 cells (IC_{50} > 1 μg/mL) (249). Carmabin A (**145**), a linear lipotetrapeptide, was isolated from the *n*-BuOH extract of *L. majuscul*a. By using the MRC-5 human embryonic lung cell line in the confluent and proliferating states (cytotoxicity assessment assay, Syntex Discovery Research), curacin A and carmabin produced the following IC_{50} values: 6.58 μg/mL (crude extract with curacin A), 0.98 μg/mL (fraction with curacin A), 0.003 μg/mL (pure curacin A); 4.8 μg/mL (crude extract with carmabin), 0.6 μg/mL (fraction with carmabin A), and 0.06 μg/mL (pure carmabin A) (250). Hexane and butanol extracts of *Symploca hydnoides* showed cytotoxic activity against HT-29 human colon cancer cells. A new depsipeptide, malevamide C (**146**), was isolated from the cyanobacterium *S. laete-viridis*, collected near the south shore of Oahu, Hawaii (251). At a concentration <2 μg/mL, this compound was found to be active against P388, A-549, and HT-29 cancer cells. The malevamide contains some unusual amino and hydroxy acids and several methylated and dimethylated residues. Other unusual moieties include 3-amino-2-methylhexanoic acid and 3-amino-2-methyl-7-octynoic acid.

Of a new series of depsipeptides, antanapeptins A–D, two [antanapeptin A (**147**) and D (**148**)] containing acetylenic acid were isolated from *L. majuscula* of the Antany Mora collection (Madagascar) (252). Both metabolites showed moderate cytotoxic activity against neuroblastoma-2A cells in mice.

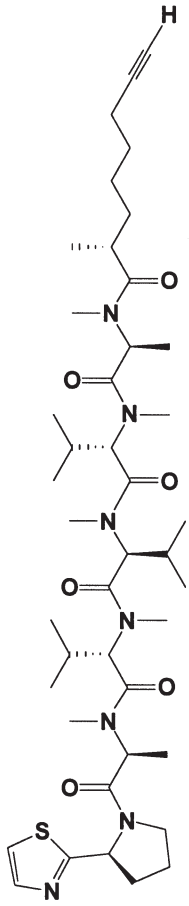
A new cyclodepsipeptide [named pitipeptolide A (**149**)], isolated from *L. majuscula* collected at Piti Bomb Holes (Guam reefs), an area known for its periodic blue-green algal blooms, appears to be uniquely distinguished in this particular collection of Dr. Valerie Paul and colleagues by the presence of a 2,2-dimethyl-3-hydroxy-7-octynoic acid residue (253). This compound exhibited weak cytotoxicity against LoVo cancer cells but possessed moderate antimycobacterial activity and stimulated elastase activity.

Yanucamides A (**150**) and B (**151**) were isolated from lipid extracts of *L. majuscula* and *Schizothrix* sp. collected at Yanuca Island (Fiji) (254). Both compounds contain a unique 2,2-dimethyl-3-hydroxy-7-octynoic acid. Ulongapeptin (**152**), a cyclic depsipeptide with a β-amino acid, 3-amino-2-methyl-7-octynoic acid, was isolated from a Palauan marine *Lyngbya* sp. The compound was cytotoxic against KB cells with an IC_{50} value of 0.63 μM (255).

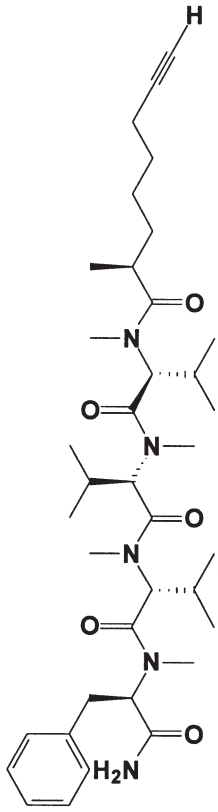
Four new depsipeptides have been isolated from the marine cyanobacterium *Lyngbya semiplena* collected from Papua New Guinea. The wewakpeptins represent an unusual arrangement of amino and hydroxy acid subunits compared with known



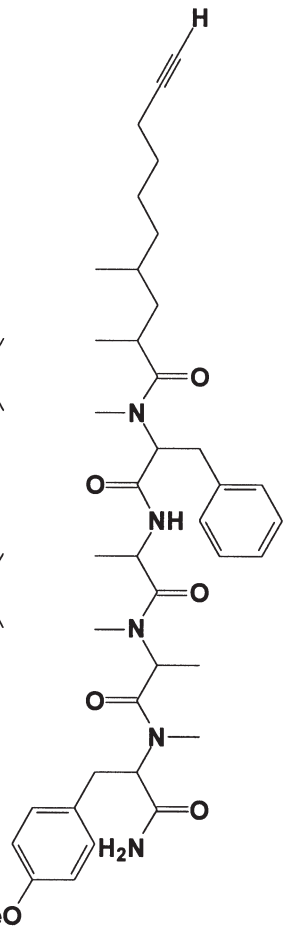
141 Apramide A, R = Me
142 Apramide B, R = H



143 Apramide G



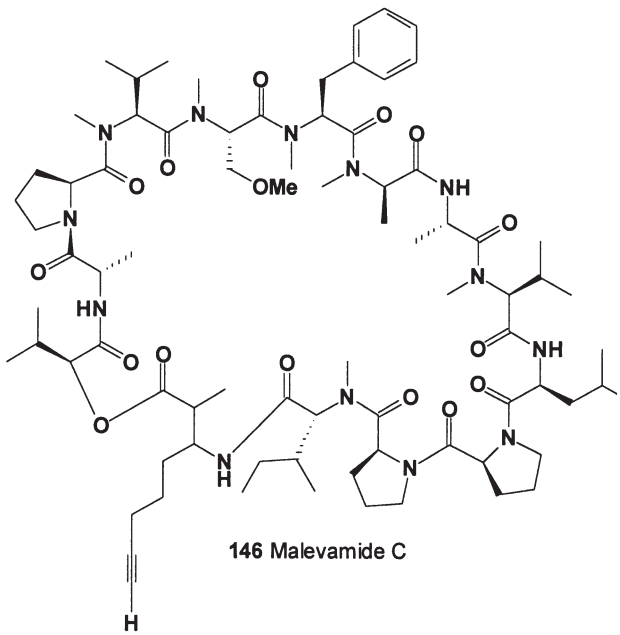
144 Dragonamide



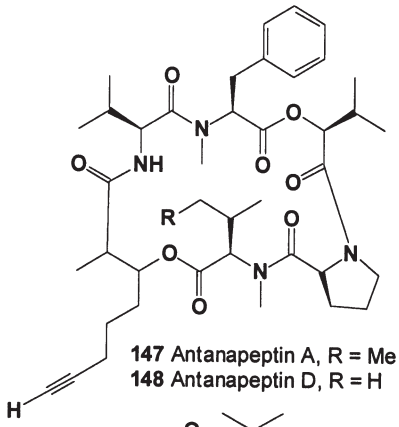
145 Carmabin A

peptides of cyanobacterial origin and possess a bis-ester, a 2,2-dimethyl-3-hydroxy-7-octynoic acid residue. Wewakpeptins A (**153**) and C (**154**) were the most cytotoxic among these four depsipeptides, with an LC_{50} value of approximately 0.4 μM for both the NCI-H460 human lung tumor and the mouse neuroblastoma-2A cell lines (256–258).

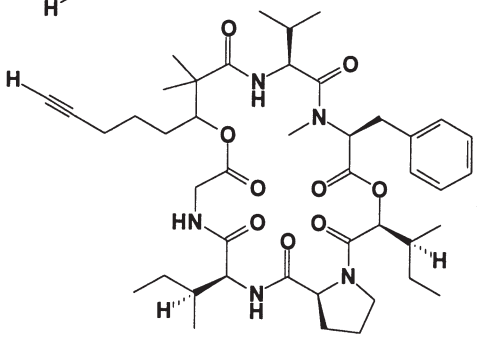
Guineamide C (**155**) is a novel cyclic depsipeptide isolated and characterized from a Papua New Guinea collection of *L. majuscula* (257). Guineamide C possesses moderate cytotoxicity to a mouse neuroblastoma-2A cell line with an IC_{50} value of 16 μM. A cyclic depsipeptide, georgamide (**156**), was isolated from an unidentified cyanobacterium (Australia) (258). Its constituent units were five amino acid residues (L-Thr, L-Pro, L-Val, N-Me-L-Val, and N-Me-L-Phe), as well as two hydroxy carboxylic acids, 2(*S*)-hydroxy-3(*R*)-methylpentanoic acid and 2,2-dimethyl-3-hydroxy-7-octynoic acid, which are also present in wewakpeptins A and B (258).



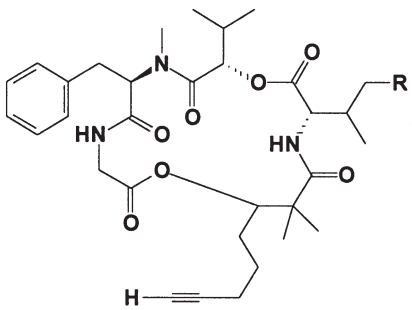
146 Malevamide C



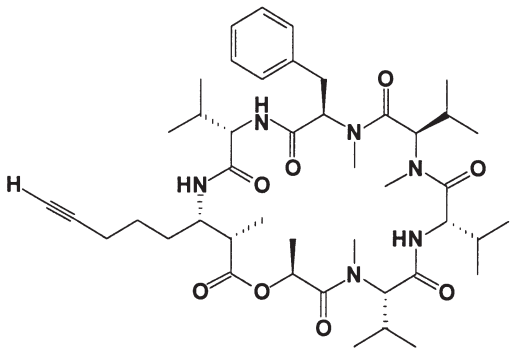
147 Antanapeptin A, R = Me
148 Antanapeptin D, R = H



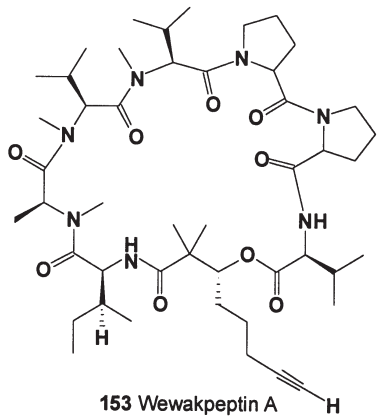
149 Pitipeptolide A



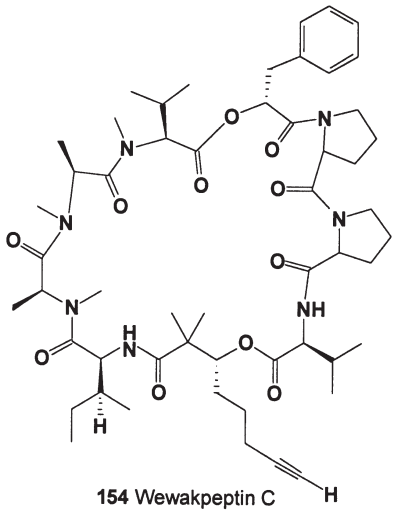
150 Yanucamide A, R = H
151 Yanucamide B, R = Me



152 Ulongapeptin



153 Wewakpeptin A



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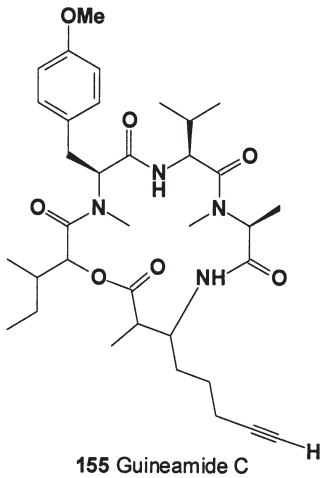
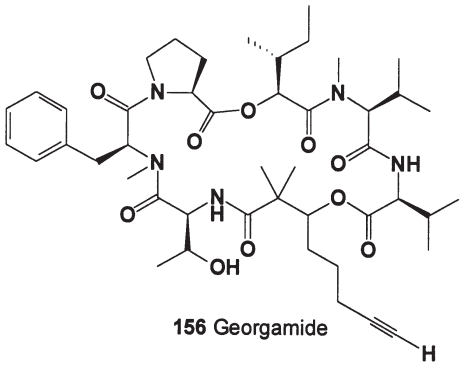


155 Guineamide C



156 Georgamide

METABOLITES OF MACRO- AND MICROALGAL SPECIES

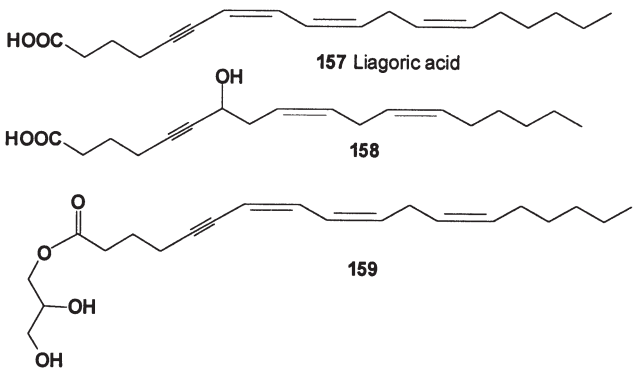
The structures of octadec-5-yne-7*Z*,9*Z*,12*Z*-trienoic acid (liagoric acid) (**157**), 7-hydroxy-5-yne-9*Z*,12*Z*-dienoic acid (**158**), and glyceryl octadec-5-yne-7*Z*,9*Z*,12*Z*-trienoate (**159**) isolated from *Liagora farinosa* are presented here. These compounds showed acute toxicity toward *Eupomacentrus leucostictus* at concentrations from 5 to 8 μg/mL. At a concentration of 31 μM, liagoric acid inhibited COX activity (259,260).

The family Caulerpaceae includes hundreds of multishaped species of marine green macroalgae, from grape-like *Caulerpa racemosa* to feathery *C. sertularioides, C. taxafolia*, and *C. mexicana,* and to the solid-bladed *C. prolifera.* Some of them are very invasive. The most devastating representative of this genus, *C. taxifolia*, has received the nickname "killer alga." Its aquarium strain was accidentally released in the wastewater from the Oceanographic Museum at Monaco, from whence it rapidly invaded the Mediterranean Sea; it is also present along the southern Californian and Australian coasts. *Caulerpa taxifolia,* like other species of *Caulerpa*, is coenocytic, representing a gigantic cell and containing millions of nuclei. The ethanolic extract of *C. prolifera* showed antitumor activity against Ehrlich ascites carcinoma *in vitro* (261). In contrast to other plants that produce a variety of toxins, but in reduced amounts, *C. taxifolia* synthesizes a single major secondary metabolite, caulerpenyne (**160**), in enormous quantities (varying from 1.9 to 10% of algal dry mass, depending on season). This sesquiterpene is toxic to herbivores, such as sea urchins, and to submarine flora.

Caulerpenyne is cytotoxic to several cell lines. In particular, it induces inhibition of SK-N-SH (neuroblastoma) cell proliferation with an IC_{50} of 10 μmol/mL after either long-time incubation (24 h) or after 2 h (262). In this respect, caulerpenyne is as efficient as other antitumor drugs, such as oxaliplatinul and paclitaxel (=Taxol). After a short incubation *in vitro*, caulerpenyne blocked polymerization of pure tubulin with an IC_{50} of 21 μM, presumably by inducing its aggregation (262). A detailed study of its antitumor efficiency and selectivity was performed on eight cancer cell lines of human origin. It produced growth-inhibitory effects in all cases, with some variability among the cell lines. Cells of colorectal cancer origin were the most sensitive to caulerpenyne, with IC_{50} values from 6.1 to 7.7 μM (263). As to the major mechanism underlying caulerpenyne cytotoxicity, available data are controversial: Both inhibition at the G2/M phase (263) and absence of the effect (262) were reported. On the other hand, the particular action of the compound on the microtubular network assayed both in the *in vitro* tubulin system and on the SK-N-SH cell line (262) could be of great importance in the future search for novel antimitotic agents.

Conventional antimitotics either stabilize (Taxol) or depolymerize (*Vinca* alkaloids) microtubules but do not induce tubulin aggregation. Tubulin may represent just one of the targets of caulerpenyne. After taking into account an early and strong cytotoxic effect of caulerpenyne, involvement of other antiproliferative mechanisms was suggested (262). Indeed, caulerpenyne was reported to block phospholipase A_2 activity and selectively inhibit stimulation of mitigen-activated kinase (MAPK) (262).

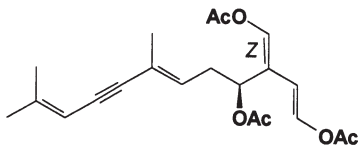
Caulerpenyne is produced in somewhat lower amounts by other Caulerpaceae and has been isolated from several *Caulerpa* species from the Mediterranean Sea (*C. prolifera*), the Pacific Ocean, and the Caribbean Sea (*C. prolifera, C. racemosa, C. lanuginosa*) (262–264). Though caulerpenyne represents a major toxic metabolite of *Caulerpa*, its numerous derivatives (**163–168**), including furocaulerpin (**169**), an acetylenic sesquiterpenoid possessing a furan ring, could contribute to the cytotoxicity of the species. It should be noted that,



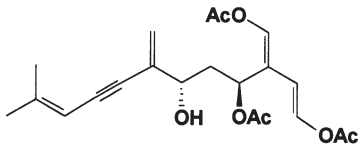
157 Liagoric acid

158

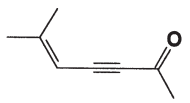
159



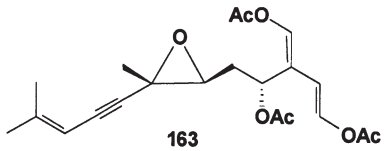
160 Caulerpenyne



161 Caulerpenynol



162 Taxifolione



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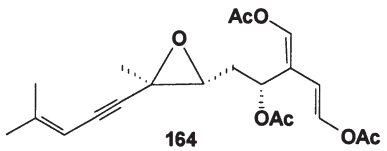

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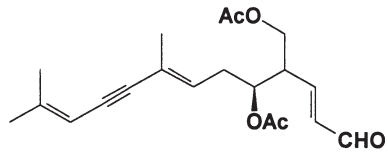
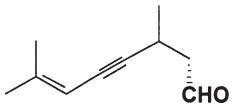
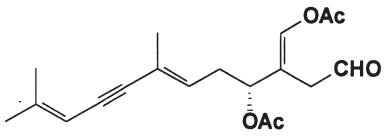


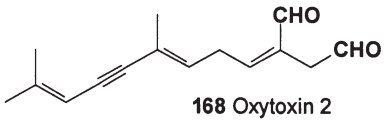
165 Taxifolial A



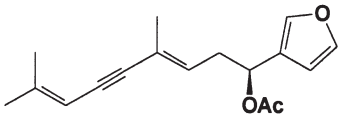
166 Taxifolial D



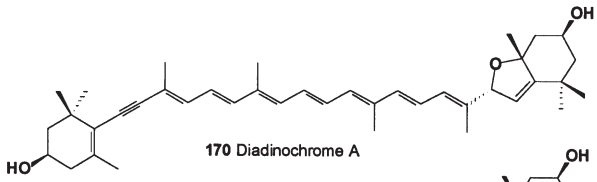
167 Oxytoxin 1



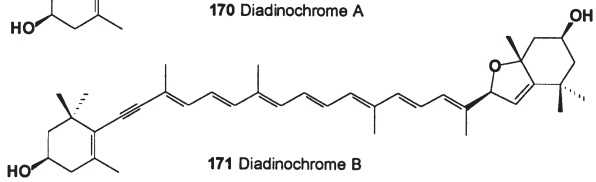
168 Oxytoxin 2



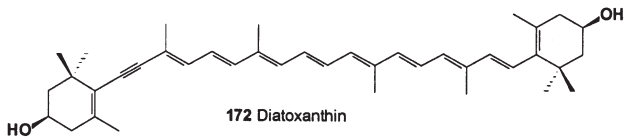
169 Furocaulerpin



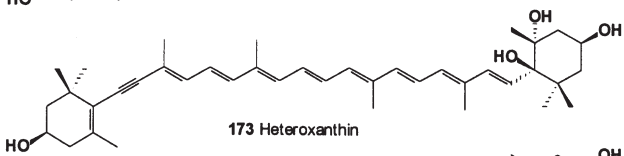
170 Diadinochrome A



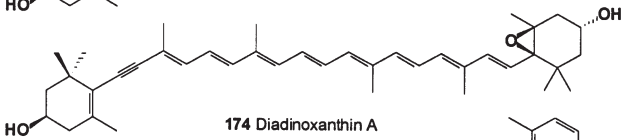
171 Diadinochrome B



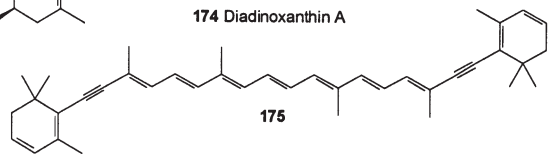
172 Diatoxanthin



173 Heteroxanthin



174 Diadinoxanthin A



175

upon wounding, *C. taxifolia* and other *Caulerpa* species, within seconds, induce transformation of caulerpenyne into highly reactive, and thus potentially more toxic, aldehydes of the oxytoxin family (265,266).

Some of the minor caulerpenyne metabolites (**161–164**) were shown to inhibit, *in vitro*, the growth of marine bacteria and marine ciliates (Protozoa) (267). The toxicity of pure compounds (**163–168**) was also evaluated on three models: mice (lethality), mammalian cells in culture (cytotoxicity), and sea urchin eggs (disturbance of cell proliferation).

These caulerpine analogs were found to be more or less toxic, with variations of efficiency depending on the assay (265,266). Bioactive furocaulerpin (**169**), an acetylenic sesquiterpenoid possessing a furan ring, was isolated from the marine alga *C. prolifera* (268).

Three carotenoids with an acetylenic unit, named diadinochromes A (**170**) and B (**171**) and diatoxanthin (= cynthiaxanthin: **172**), were isolated from the freshwater red tide organism *Peridinium bipes* (Dinophyceae). Diadinochrome A was cytotoxic to HeLa cells, whereas the other two compounds exhibited anticancer activity. Extracts of *P. bipes* exerted an inhibitory effect on the growth of *Microcystis aeruginosa* (269–271). Tsushima and co-workers (272) studied 51 carotenoids, including carotenoids with acetylenic unit(s): amarouciaxanthin B (sidnyaxanthin), crassostreaxanthin A, diatoxanthin, halocynthiaxanthin, heteroxanthin, mytiloxanthin, mytiloxanthinone, pectenols A and B, and pectenolone. Acetylenic carotenoids showed different cytotoxic activity against Raji cells (human neoplasm).

Quantitative carotenoid analysis of the microalga *Euglena viridis* revealed the presence of β,β-carotene (5% of total carotenoids), mixed with some β,ε-carotene, the β,ε-carotene-derived siphonein (siphonaxanthin 19-dodecenoate, 8%), the allenic neoxanthin (4%), and acetylenic carotenoids >86% (273–276). Those included the mono-acetylenic diatoxanthin (**172**) (major, 61%), diadinoxanthin (**174**, rearranged to diadinochrome, 12%), heteroxanthin (**173**, 1%), and the diacetylenic 3,4,7,8,3′,4′,7′,8′-octadehydro-β,β-carotene (**175**, 6%). The significance of the presence of siphonein and diacetylenic carotenoids for algal chemosystematics was briefly discussed. Heteroxanthin was also found in *Euglena gracilis* (274,275), and Xanthophyceae species (276). The principal crystallizable xanthophylls of *Tribonema aequala* were diatoxanthin, heteroxanthin, and diadinoxanthin (277).

Carotenoids of two members of the Raphidophyceae (chloromonads), *Gonyostomum semen* and *Vacuolaria virescens*, and of two tentative members of the same class (*Chattonella japonica* and *Fibrocapsa japonica)* were analyzed (278).

Group I (*G. semen* and *V. virescens*) showed a similar carotenoid pattern, comprised of diadinoxanthin (54–60% of total carotenoids), dinoxanthin (8–17%), β,β-carotene (7%), and heteroxanthin (7%), as well as neoxanthin (*G. semen*, 3%),

an epoxidic monoacetate (*G. semen*, 12%), an epoxidic carotenol, possibly 9′-*cis*-diadinoxanthin (*V. virescens*, 8%), an epoxidic diacetate (*V. virescens*, 2%), and vaucheriaxanthin 3,19-diacetate (*V. virescens*, 8%). Characteristic features common to the carotenoids encountered are a high proportion of epoxidic carotenoids (78–86%), allenic carotenoids (24–82%), acetylated carotenols (18–81%), and acetylenic carotenoids (61–67%; Group I only). The xanthophycean cultured alga *Pleurochloris meiringensis* contains heteroxanthin, diadinoxanthin, and β-carotene (279). Carotenoids extracted from freshwater red tide plankton were shown to include β-carotene (8.1%), peridinin (26.5%), dinochrome A (14.3%), dinochrome B (2.7%), dinoxanthin (1.7%), diadinochrome A (**170**, 2.7%), diatoxanthin (**172**, 6.8%), and 13′-*cis*-7′,8′-dihydroneoxanthin-20′-al 3′-β-lactoside (4.7%). Some of the isolated carotenoids were shown to be cytotoxic to mouse tumors (272).

TABLE 13
Immunosuppressive Activity of Alcohols Isolated from the Sponge *Cribrochalina vasculum* (ref. 290)

Compound	Dose (mg)	MLR (%)[a]	LCV (%)[b]	Suppression (%)
176	0.1	0	61	100
176	0.01	24	80	76
177 (15*E*)	0.1	0	34	100
177 (15*E*)	0.01	33	67	67
178	0.1	0	42	100
178	0.01	21	78	79
179	0.1	0	49	100
179	0.01	21	82	79
180 (15*E*)	0.1	0	45	100
180 (15*E*)	0.01	20	67	33

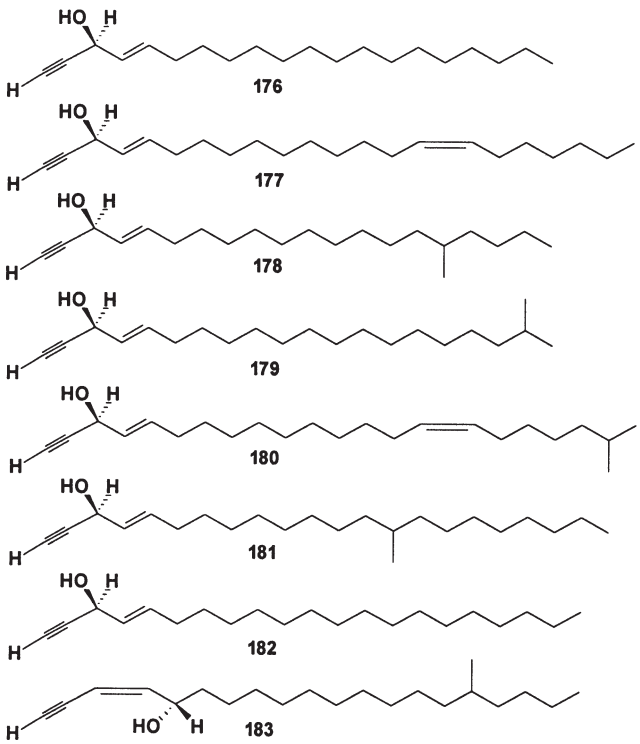
[a]Percentage of the positive (no drug) mixed lymphocyte response (MLR) control.
[b]Percentage of the positive (no drug) level control value (LCV) control.

MARINE AND FRESHWATER SPONGES (PORIFERA)

These invertebrates are quite vulnerable and are characterized by a lack of physical defenses. To resist predators and bacteria, to protect themselves from fouling, and to compete for space, they have developed effective mechanisms of chemical defense by extruding, in particular, very toxic secondary metabolites. In fact, the sponges produce the highest quantity of secondary metabolites compared with all other marine invertebrates (280–287). Some of the released compounds are of high structural complexity, exhibit unique modes of action, and are active in extremely low doses. It is no wonder that the sponges are a rich source of biologically active chemical molecules and potentially valuable pharmacological compounds. These porous organisms are known for their ability to serve as harbors for bacteria. Thus, many active compounds produced by the sponges are bactericides (more than 200 antibiotics have been isolated from this source), while others could be of bacterial origin. Intensive search for new classes of biologically active molecules led to a discovery of a series of antitumor compounds from marine sponges and the microorganisms associated with them (286,287).

More than 100 polyacetylenic metabolites have been identified in different sponge species (288), and some of these possess pronounced antitumor activities. Antitumor bioassay-guided fractionation of the organic extract of the marine Brown Bowl Sponge (*Cribrochalina vasculum)* resulted in the isolation of several closely related cytotoxic acetylenic alcohols (**176–183**) (289). Isolated compounds selected from this series showed selective *in vitro* antitumor activity against H-522 non-small cell lung line and IGROV-1 ovarian line. Five acetylenic alcohols (**176**, **177**, **179**, **180**, and **182**) with immunosuppressant and antitumor activity were isolated from the sponge *C. vasculum* and characterized (290,291). The alcohols displayed immunosuppressive activity in mixed lymphocyte reaction and CV-1 cytotoxicity assays (Table 13). In *in vitro* tests on P388 leukemia cells and on cells from human lung (A549) and colon (HT-29) tumors, these compounds had IC_{50} values that varied from 0.86 to 90 μg/mL.

Chromatographic separation of solvent-partitioned fractions of the extract of *Haliclona* sp. yielded two new compounds, halicynones A (**184**) and B (**185**), along with the known compounds triangulyne A (**186**), triangulyne E (**187**), and pellynols A (**188**), B (**189**), C (**190**), D (**191**), and I (**192**) (0.011–0.11% of dry weight). The isolated polyacetylenes were tested for cytotoxicity (292–294). Cultured HCT-116 cells were found to be very sensitive to compounds **188** (IC_{50} 0.026 μg/mL), **189** (0.12 μg/mL), **190** (0.127 μg/mL), **191** (0.103 μg/mL), and **192** (<0.008 μg/mL), whereas the acetylenic ketones **184** and **185** were not effective (IC_{50} > 78 μg/mL). The unusually high cytotoxicities of **188–192** and lack of activity of **184** and **185** suggest that not only a relatively rigid, rod-like structure of the



molecule but also the presence of 1-yn-3-ol were required for this type of biological activity of the above compounds. Pellynols A–D, I, and F (**193**), having a terminal 1-yn-3-ol, showed strong cytotoxicity against several melanoma and ovarian cancer cell lines (IC_{50} 0.08–2.0 μg/mL) (293,294). Pellynic acid (**194**) inhibited inosine monophosphate dehydrogenase with an IC_{50} of 1.03 pg/mL.

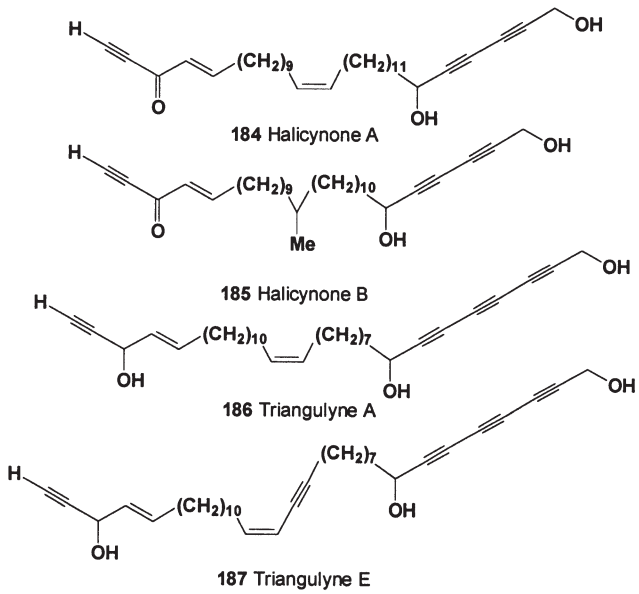
Acetylenic alcohols, strongylodiols A–D (**195–198**), were obtained from the Okinawan marine sponge belonging to the genus *Strongylophora*. Each of these compounds was an enantiomeric mixture in a different ratio and showed cytotoxic activity toward human T lymphocyte leukemia (MOLT-4) cells (295,296).

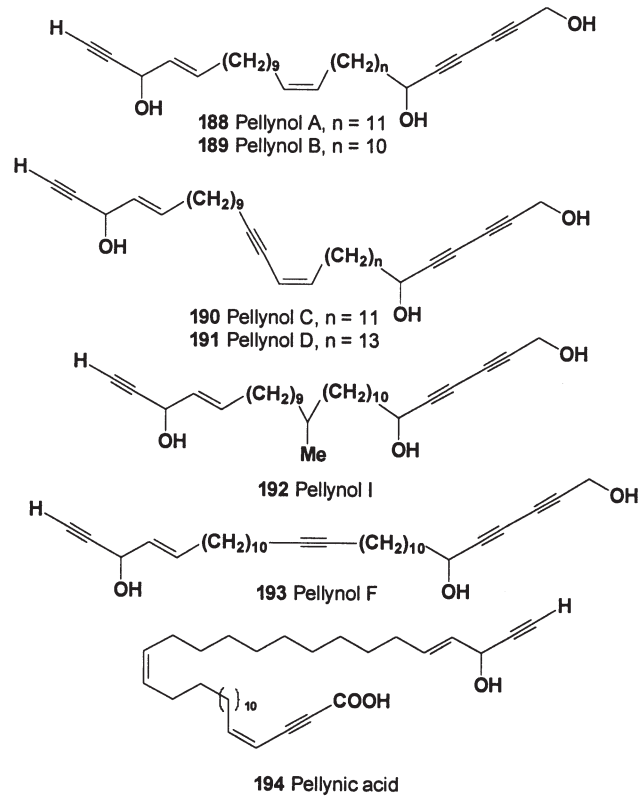
Polyacetylenes with cytotoxic activities against human tumor cell lines (A549, SK-OV-3, SK-MEL-2, XF498, and HCT15) have been isolated from the marine sponge *Petrosia* sp. and given the trivial names of dideoxypetrosynols A–D (**199–202**) (297,298). Compound A (**199**) inhibited DNA replication (299), and a mechanism of its action on cultured human SK-MEL-2 skin melanoma cells has been suggested (300). It is worthy of mention that dideoxypetrosynols B (**200**), D (**201**), and C (**202**) revealed, depending on the test used, cytotoxic activities one order of magnitude higher than those found for doxorubicin (Table 14).

Lembehyne A (**203**), a novel long-chain polyacetylene, was isolated from the Indonesian marine sponge *Haliclona* sp. (301). Lembehyne A induced bipolar neuritogenesis of Neuro-2A cells at 1 μg/mL. Acetylcholinesterase activity of Neuro-2A was also increased by treatment with **203**. Furthermore, the cell cycle of Neuro-2A cells was specifically blocked by **203** at the G1 phase. Lembehynes B (**204**) and C (**205**), which possess different types of long carbon-chain parts compared with that of lembehyne A, also exhibited neuritogenic activity against a neuroblastoma cell line, Neuro-2A. This indicates the importance of a particular stereochemistry (presence of a hydroxyl group at C-3) of lembehynes for the revealed activity (302–304). A new long-chain acetylene named fulvinol (**206**) was isolated from the sponge *Reniea fulva* from Algeciras Bay (Spain), and it exhibited cytotoxicity against four tumor cell lines (ED_{50} = 1 μg/mL) (305).

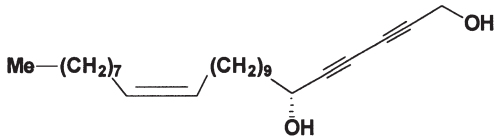
Adociacetylenes A–D (**207–210**) were isolated as new polyacetylenes from the Okinawan marine sponge *Adocia* sp. (306). Adociacetylenes A, C, and D exhibited inhibitory activity in the *in vitro* endothelial cell-neutrophil leukocyte adhesion assay. All acetylenes were highly cytotoxic to P388, A-549, HT-29, and MEL-28 melanoma cells.

Two acetylenic compounds, petrosynol (**211**) and petrosolic acid (**212**), from the Red Sea sponge *Petrosia* sp. inhibited reverse transcriptase of HIV virus (307,308). The sponge *Strongylophora durissima* yielded two new acetylenic derivatives, durissimols A and B (**213**), and duryne (**214**) (309–311). Among them, durissimol B and duryne showed potent cytotoxicity against human gastric tumor (NUGC) cells. Duryne, a cytotoxic metabolite that inhibits the growth of both mouse and human tumor cell lines *in vitro,* was previously isolated from the marine sponge *Cribrochalina dura* (310,311).

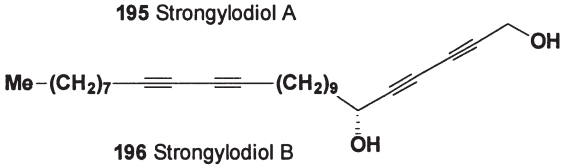




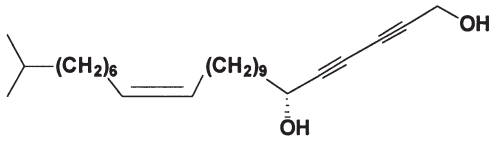
A new C_{43} acetylenic alcohol, vasculyne (**215**), was isolated by cytotoxicity-guided fractionation of the Caribbean sponge *Cribrochalina vasculum* (312). Vasculyne yielded average GI_{50}, TGI, and LC_{50} values of 0.2, 0.7, and 6.7 mg/mL, respectively (where GI_{50} is the concentration required for 50% inhibition of cancer cell growth and TGI is the concentration required for total inhibition of cancer cell growth), and exhibited



195 Strongylodiol A



196 Strongylodiol B



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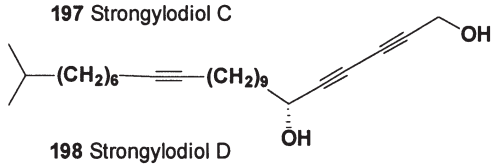
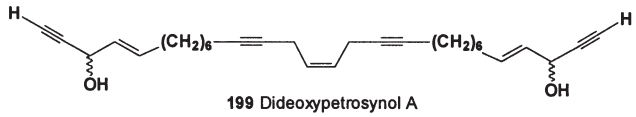
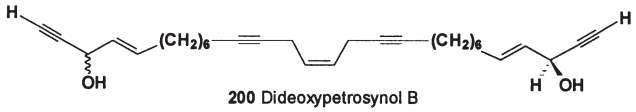


198 Strongylodiol D



199 Dideoxypetrosynol A



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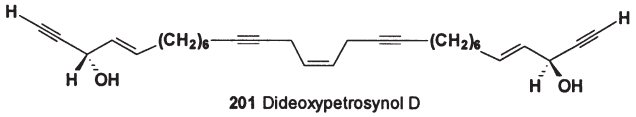
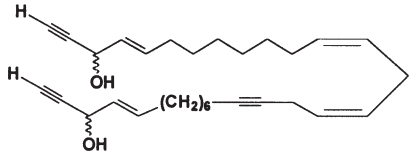


201 Dideoxypetrosynol D



202 Dideoxypetrosynol C

modest differential cytotoxicity toward the melanoma and colon tumor cell-line subpanels when determined for a panel of 60 human tumor cell lines, representing nine tumor tissue types, used by the National Cancer Institute (NCI). Structurally, **215** is closely related to the C_{30} compound duryne, previously isolated from the Caribbean sponge *C. dura.*

Osirisynes A–F (**216–221**, respectively), highly oxygenated C_{47} polyacetylenes, have been isolated from the sponge *Haliclona osiris* collected from Guam (313,314). These compounds are characterized by the presence of a diacetylenic carbinol and an α-acetylenic carboxylic acid. Osirisynes A–F exhibited moderate cytotoxicity against a human leukemia cell-line (KS62), with LC_{50} values of 25, 48, 52, 25, 20, and 22 μM for **216–221**, respectively. In addition, **218**, **220**, and **221** inhibited

TABLE 14
***In vitro* Cytotoxicities[a] of Dideoxypetrosynols Against Human Solid Tumors[b]**

Compound	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
199	1.43	0.02	0.01	0.16	0.17
200	1.98	0.21	0.11	1.83	1.56
201	12.41	1.83	1.27	1.83	1.87
202	5.78	0.02	0.02	3.02	1.94
Doxorubicin	0.09	0.16	0.11	0.13	1.02

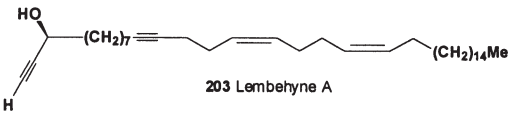
[a]ED_{50}, μg/mL. From Reference 300. For abbreviation see Table 3.
[b]A549, human lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human skin cancer; XF498, human central nervous system cancer; HCT15, human colon cancer. Doxorubicin, also known as adriamycin, is one of the most commonly used chemotherapeutic drugs and exhibits a wide spectrum of activity against solid tumors, lymphomas, and leukemias.

Na^+/K^+-ATPase and reverse transcriptase at concentrations of l μg/mL.

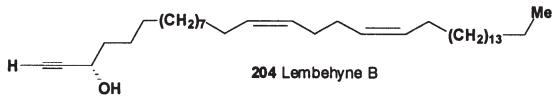
A novel acetylenic compound, taurospongin A (**222**), was isolated from the Okinawan marine sponge *Hippospongia* sp. (315), and amino acid analysis of the hydrolysis products of **222** showed the presence of taurine. Two fragments, trihydroxylamide and an unsaturated fatty acid methyl ester, were obtained by methanolysis to elucidate the lengths of the methylene chains. Metabolite **222** showed inhibitory activity against c-erbB-2 kinase (IC_{50} 28 μg/mL).

Callyspongamide A (**223**), a cytotoxic polyacetylenic amide, has been isolated from the marine sponge *Callyspongia fistularis* collected in the Red Sea. Callyspongamide A is an amide derivative of a C_{17}-polyacetylenic acid and phenethylamine. It showed a moderate cytotoxicity against HeLa cells with an IC_{50} value of 4.1 μg/mL (316).

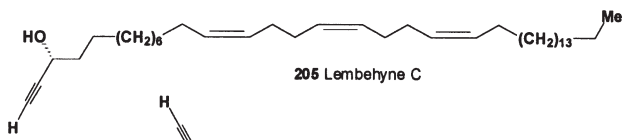
Investigation of the organic extract of a Red Sea sponge, *Callyspongia* sp., resulted in the isolation and identification of



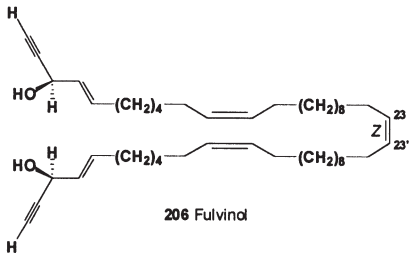
203 Lembehyne A



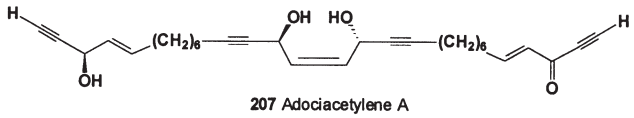
204 Lembehyne B

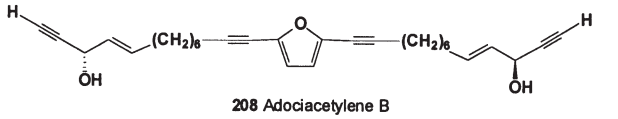


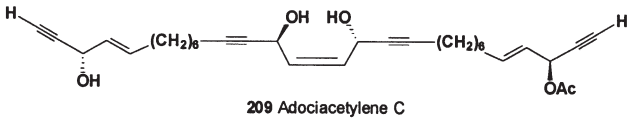
205 Lembehyne C

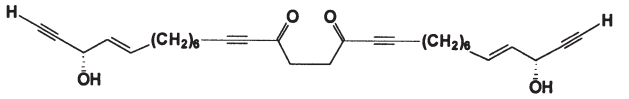


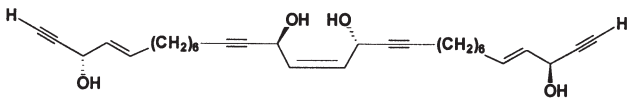
206 Fulvinol

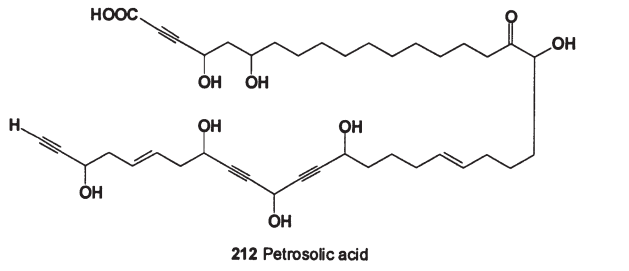




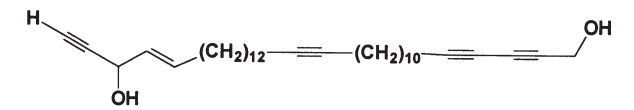


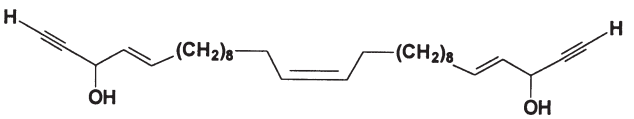


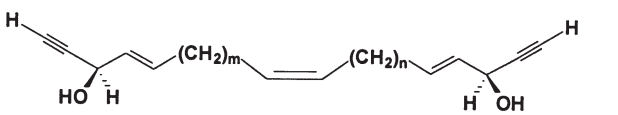


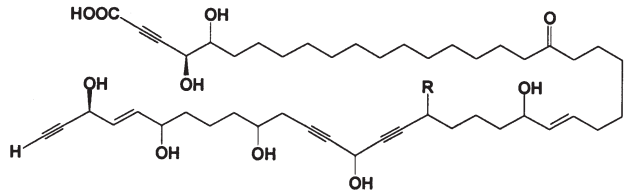


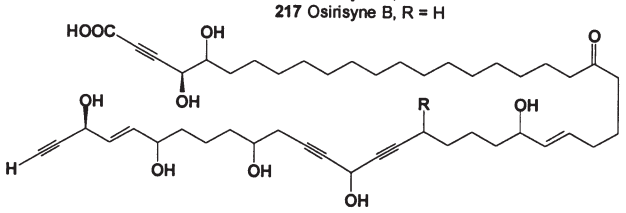
three new C_{22}-polyacetylenic alcohols, callyspongenols A–C (**224–226**), together with siphonchalynol (**227**) (317,318). All compounds showed moderate cytotoxicity against P388 and HeLa cells. The cytotoxicity of compounds **224–227** against HeLa and P388 cells is presented in Table 15. Previously, siphonochalynol (**227**) was isolated from the sponge *Siphonochalina* sp. (318).

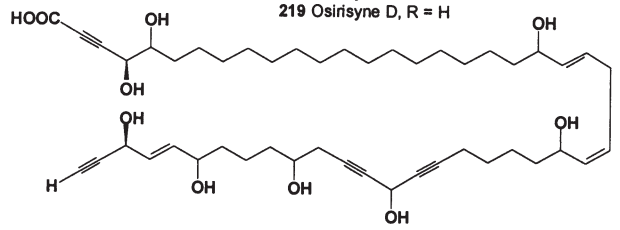


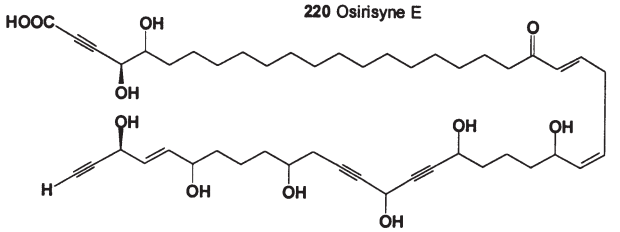












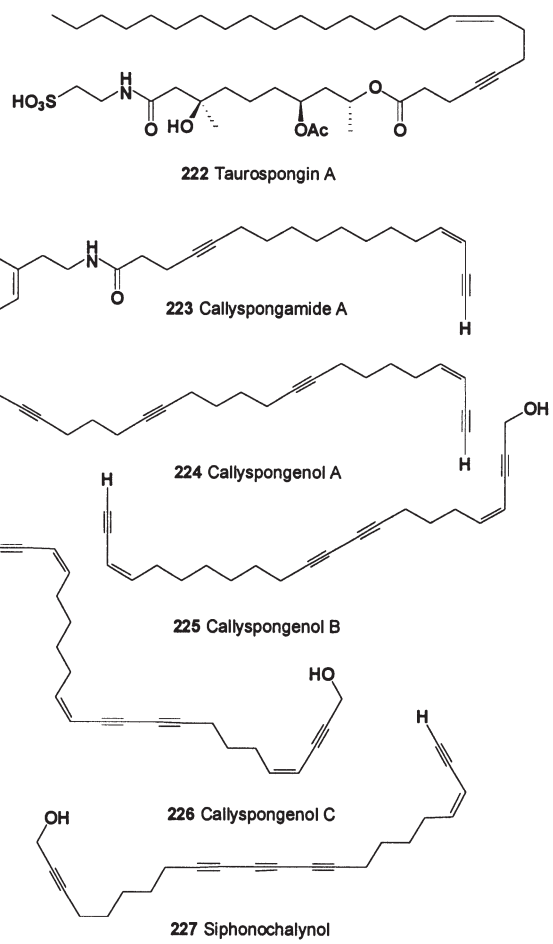
Three new acetylenic metabolites (**228–230**) were isolated from the sponge *Stelletta* sp. collected from Gagu-Do, Korea (319). These compounds exhibited no significant antimicrobial activity and displayed only weak cytotoxicity against the human leukemia cell-line K562 with LC_{50} values of 43.5, 51.3, and 62.5 µg/mL for **228**–**230**, respectively.

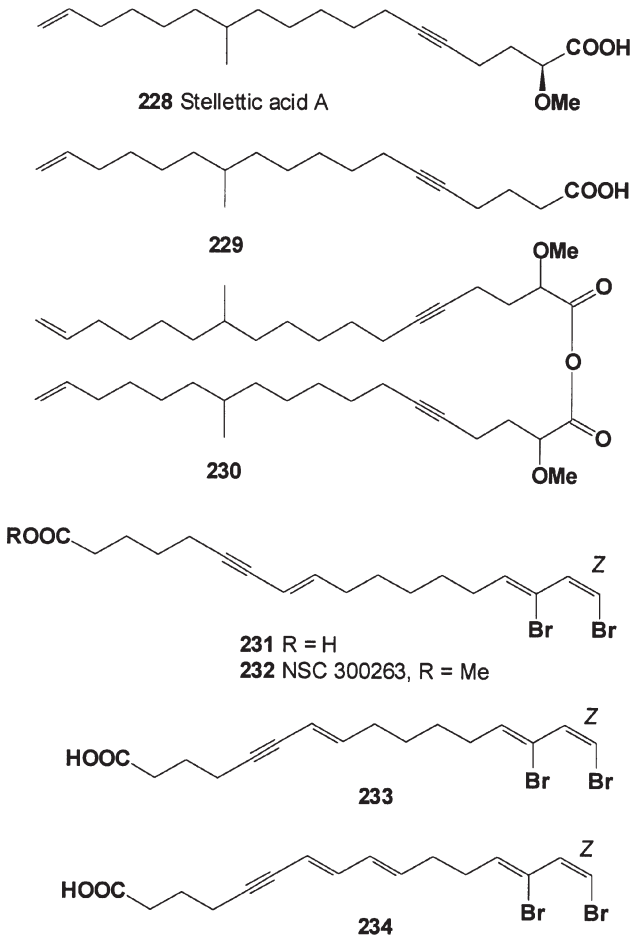
(7*E*,13ξ,15*Z*)-14,16-Dibromo-7,13,15-hexadecatrien-5-ynoic acid (**231**) and its methyl ester (**232**), also known as NSC 300263, were isolated from the sponge *Xestospongia muta*. The ED values of **231** for 50% inhibition in *in vivo* PS and L-1210 cell culture evaluations were 24 and 34 µg/mL, respectively, and the corresponding doses of **232** were 29 and 34 µg/mL (320). Similar brominated fatty acids (**233** and **234**)

TABLE 15
Cytotoxicity[a] of Compounds Against P388 and HeLa Cells

Compound	**224**	**225**	**226**	**227**	Adriamycin[b]
P388	2.2	10.0	2.2	2.2	0.04
HeLa	4.5	10.0	3.9	5.1	0.066

[a]IC_{50}, µg/mL From Reference 317. For abbreviation see Table 2.
[b](Also known as doxorubicin). Positive cytotoxicity control.





were isolated from an Indonesian sponge, *Oceanapia* sp. (321). Their common structural feature is a (13*E*,15*Z*)-14,16-dibromodiene terminus. Both compounds are unstable oils. The mixture exhibits mild cytotoxicity toward KB cells. A methanol-soluble extract of the frozen marine sponge *Petrosia* sp. showed significant activity in the brine shrimp larvae lethality bioassay (LD_{50} 30 μg/mL), and cytotoxic activities against a panel of human solid-tumor cells. A number of very long chain C_{46} polyacetylenic alcohols from the marine sponge *Petrosia* sp., named petrocortynes (**235–240**), have been identified. These compounds showed different cytotoxic activities human tumor cells (Table 16) (322).

Acetylenic enol ethers of glycerols, including bioactive compounds **241–246**, have been isolated from a sponge of the genus *Petrosia*. Compounds **241** and **243** exhibited weak cytotoxicity against a human leukemia cell-line (K-562) (323). Bioactivities of glyceryl enol ether compounds **241, 242,** and **244**, of the yne-diene series, exhibited weak cytotoxicity against the human leukemia cell-line K-562 (LC_{50} 9.2, 57, and 29 μg/mL for **214, 242,** and **244**, respectively), whereas **244–246**, possessing the yne-ene group, were less active (LC_{50} > 100 μg/mL).

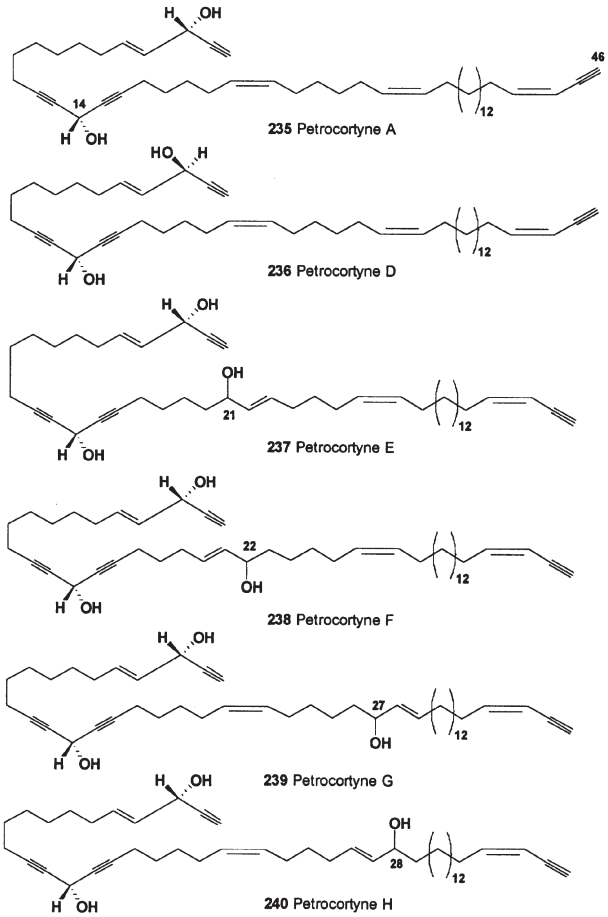
The marine sponge *Prianos osiros*, from Pohnpei (Micronesia), yielded a new cytotoxic acetylenic carotenoid, 3,3′,5,19′-tetrahydroxy-7′,8′-didehydro-γ,ε-carotene-8-one (**247**) (324), which was cytotoxic toward cultured human colon tumor cells, HCT 116 (IC_{50} 4.38 μg/mL). Two new carotenoids, the neoplasm inhibitors 19-hexanoyl-oxymytiloxanthin (**248**) and 19-butanoyloxymytiloxanthin (**249**), have been isolated from the marine sponge *Phakellia stelliderma* collected in Okinawa. Both compounds showed mild cytotoxic activity against P388 mouse leukemia cells (325).

Callipeltoside A (**250**), the first member of a novel class of

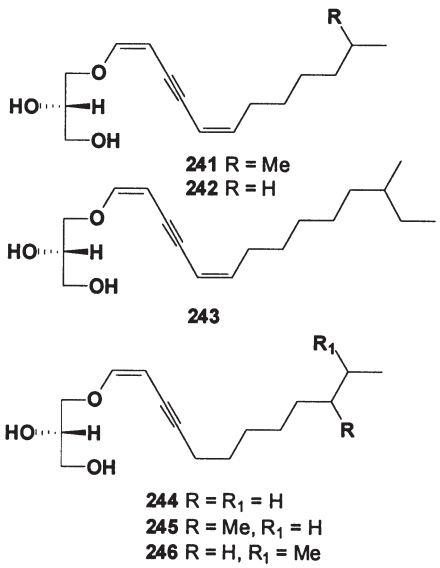
TABLE 16
Cytotoxic Activity[a,b] of Petrocortynes Isolated from the Marine Sponge *Petrosia* sp.

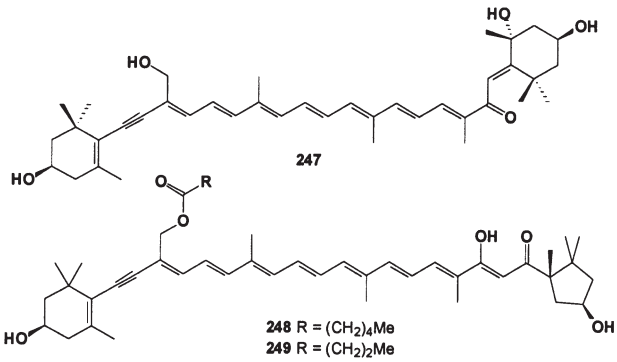
Compound	A549	SK-OV3	SK-MEL2	XF498	HCT15
235	1.1	0.6	1.1	1.7	1.0
236	1.6	0.5	0.9	1.7	1.0
237	1.7	2.2	1.9	>3.0	3.7
238	1.3	0.1	0.1	0.6	0.8
239	>3.0	>3.0	>3.0	>3.0	>3.0
240	1.4	0.1	0.2	1.2	1.2
Doxorubicin	0.1	0.2	0.2	0.2	0.9

[a]ED_{50}, μg/mL. From Reference 322. For abbreviation see Table 3.
[b]A549; human lung carcinoma; SK-OV-3; human ovarian cancer; SK-MEL-2; human skin cancer; XF498; human CNS cancer; HCT15; human colon cancer.



marine glycoside macrolides, was isolated from the lithistid sponge *Callipelta* sp. by Minale and co-workers in 1996 (326). Preliminary biological assays indicated that this marine natural product exhibited cytotoxic activity against NSCLC-N6 human





bronchopulmonary non-small-cell lung carcinoma and P388 cell lines. Callipeltosides A (**250**), B (**251**), and C (**252**) are moderately cytotoxic against NSCLC-N6 cells, with IC_{50} values of 10.0, 15.1, and 30.0 µg/mL, respectively (327).

A novel macrolide, spongidepsin (**253**), has been isolated from the Vanuatu marine sponge *Spongia* sp. (328). The structure of **253** contains 9-hydroxy-2,4,7-trimethyltetradeca-14-ynoic acid and *N*-methyl-phenylalanine residues joined in a 13-membered ring. Spongidepsin showed cytotoxic activity against J774.A1, WEHI-164, and HEK-293 cancer cell lines, with an IC_{50} value in the submicromolar range (see Table 17).

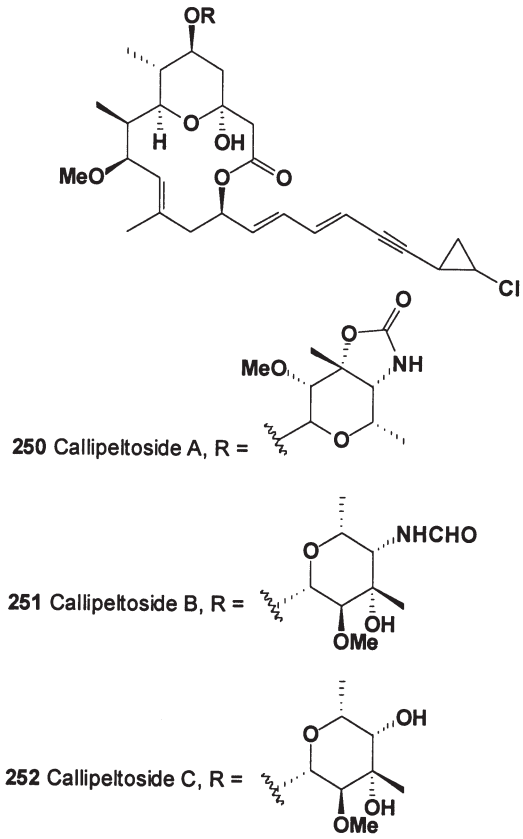
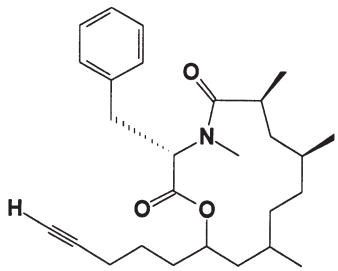


TABLE 17
***In vitro* Antiproliferative Activity of Spongidepsin (253, ref. 328)**

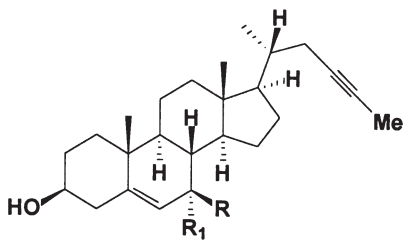
Cell lines	Spongidepsin IC_{50} (μM)	6-Mercaptopurine IC_{50} (μM)
J774.A1	0.56	0.003
HEK-293	0.66	0.007
WEHI-164	0.42	0.017

[a]For abbreviation see Table 2.

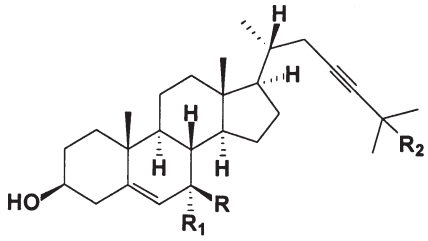
New acetylenic sterols, gelliusterol A (**254**: 26,27-bisnorcholest-5-en-23-yn-3-β-7,α-diol), its corresponding 7-ketone, gelliusterol B (**255:** 26,27-bisnorcholest-5-en-23-yn-3-β-ol-7-one), and gelliusterols C (**257**: cholest-5-en-23-yn-3-β-7-one) and D (**258**: cholest-5-en-23-yn-3-β-25-diol-7-one), were isolated from an unidentified species of sponge, *Gellius* sp. (329). Biological evaluation of gelliusterols A, B, and C was performed on cancer cell lines P388, HT-29, A-549, DU-145, and MEL-28. Gelliusterols A and B exhibited moderate activity, with IC_{50} values greater than 1 μg/mL. An activity of 0.5 μg/mL was observed with gelliusterol C against HT-29, while the other cell lines gave IC_{50} values above 1 μg/mL. The quantity of gelliusterol D (**258**) and **259** was insufficient for biological testing (330,331). Previously, bioactive compound (**256**) was isolated from sponge *Calyx podatypa* by Doss and Djerassi (332).



253 Spongidepsin



254 Gelliusterol A, R = H, R_1 = OH
255 Gelliusterol B, R,R_1 = O
256 R = R_1 = H



257 Gelliusterol C, R, R_1 = O, R_2 = H
258 Gelliusterol D, R,R_1 = O, R_2 = OH
259 R = R_1 = R_2 = H

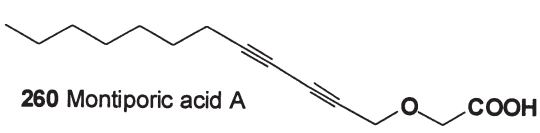
SOFT CORALS AND OTHER COELENTERATES

The old phylum Coelenterata, now included in the phylum Cnidaria, contains the corals, jellyfish, hydras, and sea anemones. Soft corals can be found worldwide in tropical environments, and they can contain symbiotic dinoflagellate algae (Zooxanthellae) that provide the coral with food in return for a safe environment. The defense of soft corals is based mainly on their stinging cells. At the same time, some fish and molluscs are attracted by the fleshy body of the soft corals. Thus, they release poisonous secondary metabolites (mostly terpenes) to deter potential predators, as well as to protect themselves from algal and bacterial colonization. In addition, the soft corals are territorial and will defend their territory by releasing toxic compounds inhibiting the growth of neighboring animals and algae (333–338). Some corals' secondary metabolites showed antibacterial, antifungal, cytotoxic, and anticancer activities.

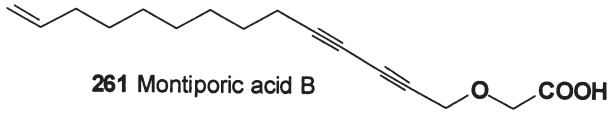
The genus *Montipora* is very rich in acetylenic compounds, and many of them are cytotoxic and/or possess antifungal and antibacterial properties. Two polyacetylene carboxylic acids, montiporic acids A (**260**) and B (**261**), were isolated from the eggs of the scleractinian coral *M. digitata* (339). Montiporic acids A and B were not only antibacterial against *Escherichia coli* but also cytotoxic against P388 murine leukemia cells, with IC_{50} values of 5 and 12 μg/mL, respectively.

Coral metabolites **262–275** and four known diacetylenes (**260**,**261**) have been isolated from the methanolic extract of the stony coral *Montipora* sp. (340). The compounds exhibited significant cytotoxicity against a small panel of human solid tumor cell lines (see Table 18). Compounds **262–270** appear to share a common 2,4-diyne moiety as a biosynthetic precursor. Compounds **271–275** are similar to compounds **262–270** in having a diyne group, but the position at which it is found is different. 2,4-Diynes are encountered more frequently in corals, and this may raise a question of the origin of **271–275**.

Compound **271** showed significant cytotoxicity against human skin cancer and human ovarian cancer cell lines. In gen-



260 Montiporic acid A

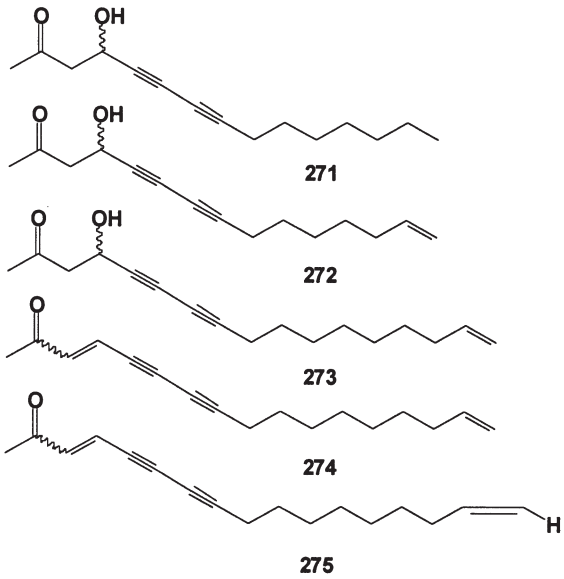


261 Montiporic acid B

TABLE 18
Cytotoxic Activities[a] of Compounds Against Human Solid Tumor Cells[b]

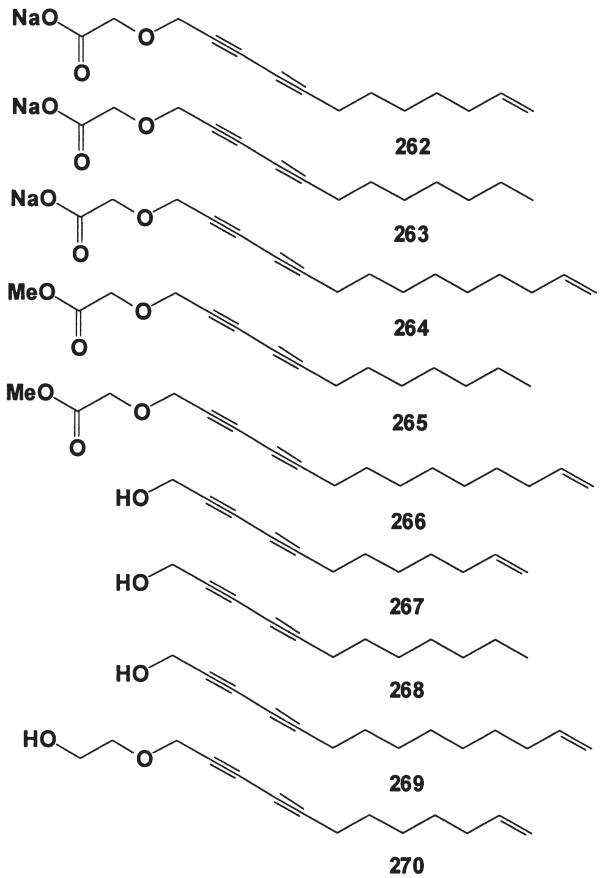
Compound	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
262	>30	>30	>30	>30	>30
263	6.31	7.50	7.97	7.72	8.30
264	6.26	4.88	4.68	4.96	4.47
265	>30	20.52	>30	>30	25.61
266	>30	>30	>30	>30	>30
267	13.78	9.79	9.56	10.78	12.93
268	5.48	4.63	4.45	5.59	5.90
269	3.90	3.23	3.94	5.26	3.32
270	22.73	17.94	25.08	16.88	24.05
271	4.17	1.81	1.40	3.70	3.73
272	4.97	3.85	3.74	3.87	3.42
273	4.91	3.34	3.52	4.45	4.18
274	6.39	3.52	4.21	5.50	4.56
275	>30	5.23	4.61	29.16	11.30

[a]ED_{50}, μg/mL. From Reference 340. For abbreviation see Table 3.
[b]Key to cell lines used: A549 = human lung cancer; SK-OV-3 = human ovarian cancer; SK-MEL-2 = human skin cancer; XF498 = human CNS cancer; HCT15 = human colon cancer.



eral, diacetylenes with the α-hydroxy ketone functionality (**271**-**273**) were more active. The *trans*-isomer (**274**) was more active than the *cis*-isomer (**275**), as in the case of montiporynes A–D (**276–279**). Montiporyne A (**276**), an analog of **275**, showed significant cytotoxicity toward human solid tumor cell lines and cell cycle inhibition of the HCT116 cell.

Six acetylenic compounds, montiporynes A–F (**276–281**, respectively), with cytotoxic activities against human solid tumor cell lines SK-OV-3, SK-MEL-2, XF498, and HCT15, have been isolated from the stony coral *Montipora* sp. (Table 19) (341). Polyacetylenes **282–285**, found in three species of the hermatypic corals, *Montipora* sp., *M. mollis*, and *Pectinia lactuca*, represent metabolites of coelenterates that exhibit ichthyotoxicity and inhibit the growth of some bacteria, fungi, and fish (342).



MARINE AND FRESHWATER MOLLUSKS

The mollusks (=molluscs) are members of the large and diverse phylum Mollusca, which includes eight living classes: Caudo-

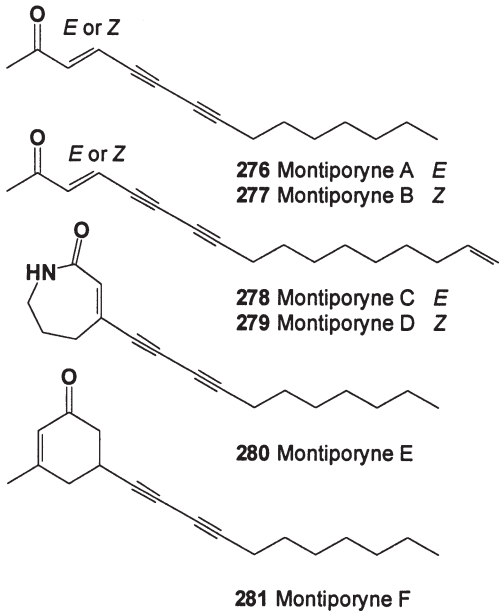


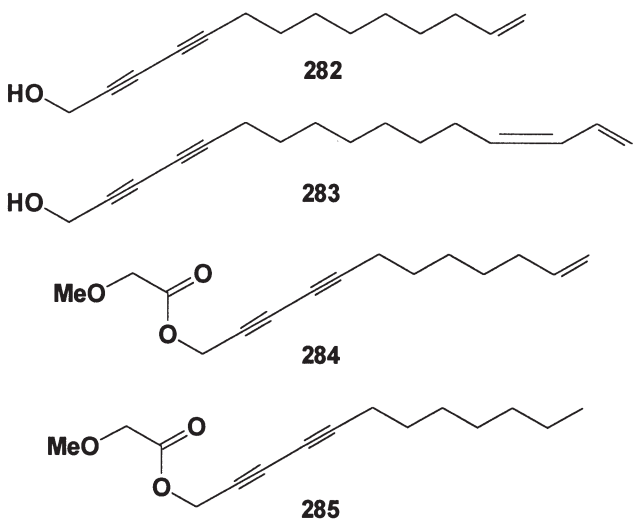
TABLE 19
***In vitro* Cytotoxicities[a] of Montiporynes Against Human Solid Tumor Cells[b]**

Compound	A549	SK-OV3	SK-MEL2	XF498	HCT15
276	>50	3.2	1.4	1.9	3.7
277	>50	2.5	1.5	3.2	5.2
278	>50	25.9	42.6	>50	>50
279	>50	45.1	43.1	>50	>50
280	>50	>50	>50	>50	>50
281	>50	29.2	36.7	31.3	45.1
Cisplatin	0.6	0.9	0.7	0.6	0.6

[a]ED_{50}, μg/mL. From Reference 341. For abbreviation see Table 3.
[b]A549, human lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human skin cancer; XF498, human CNS cancer; HCT15, human colon cancer. Compounds were assayed in two separate batches.

foveata (deep-sea wormlike creatures), Aplacophora (solenogasters, deep-sea wormlike creatures), Polyplacophora (chitons), Monoplacophora (deep-sea limpet-like creatures), Scaphopoda (tusk shells), Cephalopoda (squids, octopuses, nautilus, and cuttlefish), Bivalvia (clams, oysters, scallops, and mussels), and Gastropoda (nudibranchs, snails and slugs, limpets, and sea hares) (343,344). Only the two last classes (Bivalvia and Gastropoda) have been well studied and have been of great interest from the pharmaceutical point of view (345,346).

The ethanol extract of the mucous secretion from the opisthobranch mollusk *Oxynoe olivacea* was examined and found to contain two novel ichthyotoxic metabolites, oxytoxins 1 (**167**) and 2 (**168**) (347,348). The structures of the two compounds are closely related to the metabolites previously isolated from the alga *Caulerpa prolifera*. The activity of the most stable compound was studied to investigate the possibility of a further biological role for these metabolites, which represent an uncommon example of bioactive molecules produced *in vivo* from a dietary precursor. Another acetylenic bioactive metabolite, caulerpenyne (**160**), was isolated from the mesogastropod *Littorina irrorata*, which used the macrophytes



Caulerpa prolifera and *Cymopolia barbata* for its diet (348).

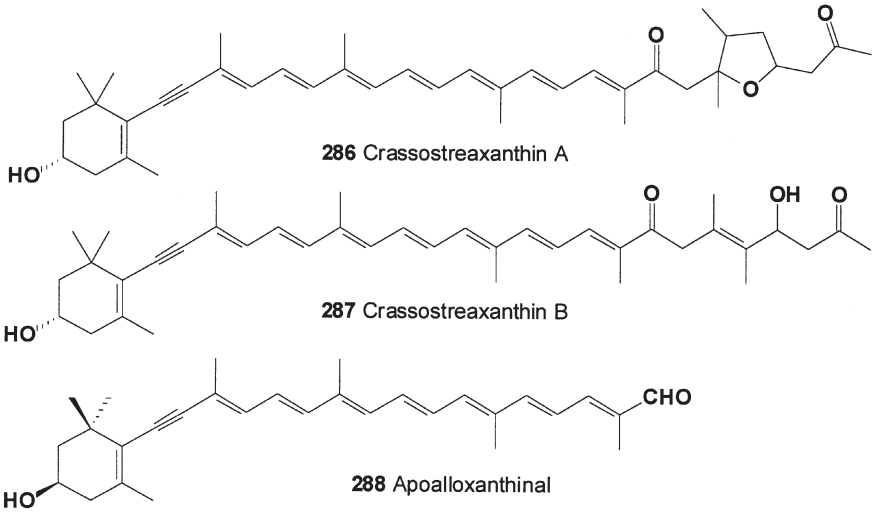
Cytotoxic carotenoids, named crassostreaxanthins A (**286**) and B (**287**) and apoalloxanthinal (**288**), were isolated from the oyster *Crassostrea gigas* (Ostreidae) (349), and apocarotenoid **292** was isolated from the marine shellfish *Mytilus coruscus* (350). Acetylenic carotenoids [**172, 173, 289–291**, 7,8-didehydro-β-cryptoxanthin (**292**), 6-epi-heteroxanthin (**293**), and **294**] have been isolated from three species of Chinese freshwater corbicula clams (*Corbicula japonica*, *C. sandai*, and *Corbicula* sp. (351).

The antimutagenic and anticancer effects of carotenoids (β-carotene, cynthiaxanthin (= diatoxanthin), astaxanthin and halocynthiaxanthin, mytiloxanthin, and halocynthiaxanthin) isolated from marine and freshwater bivalve mollusks *Crassostrea gigas* and *Corbicula fluminea* against *S. typhimurium* (TA98) and cancer NCI-H87, HeLa, HT-29, MG-63 cell lines were reported (352).

Several cytotoxic acetylenic carotenoids (**172, 173, 289, 291**), isomytiloxanthin (**295**), 19′-(hexanoyloxy)-isomytiloxanthin (**296**), hydroamarouciaxanthin B (**297**), pyrrhoxanthinol (**298**), and halocynthiaxanthin (**299**) have been identified from muscle of *Mytilus edulis* (353–357). Diatoxanthin (**172**), alloxanthin (= pectenoxanthin: **289**), and pectenolone from *Pectene maximus* (358) and *Patinopectene yessoensis* (359), pectenols A and B from *Mytilus coruscus* (360), crassostreaxanthins A and B (**286** and **287**) from *Crassostrea gigas* (361), and a series of carotenoids with a 5,6-dihydro-β-end group from *Fusinus perplexus* (362) have been reported as the principal carotenoids in marine shellfish. Carotenoids in eight species of freshwater and sea mollusks from Russia were investigated (363). The following compounds have been isolated from the named organisms: alloxanthin, mytiloxanthin, isomytiloxanthin, and the halocynthiaxanthin ether from *Modiolus modiolus* and *Crenomytilus grayanus*; alloxanthin and mytiloxanthin from *Mytilus galloprovincialis*; alloxanthin, mytiloxanthin, isomytiloxanthin, halocynthiaxanthin ether, and pectenolone from *Mizuhopecten yessoensis* have been isolated (363,364).

Marine depsipeptides are natural bio-oligomers composed of hydroxy and amino acids linked by amide and ester bonds; many of them show very promising biological activities, including anticancer, antibacterial, antiviral, antifungal, anti-inflammatory, anticlotting, and antiantherogenic properties. Depsipeptides have shown the greatest therapeutic potential as anticancer agents (365–367).

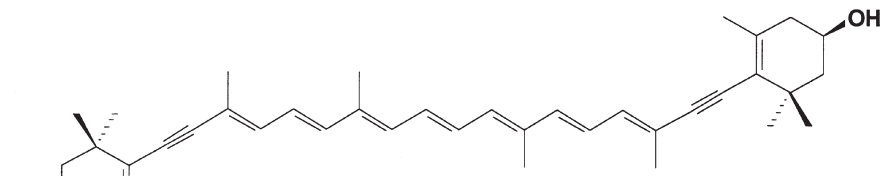
The depsipeptides onchidin (**300**) and onchidin B (**301**) were isolated from the pulmonate mollusk *Onchidium* sp. Onchidin contains the β-amino acid, 3-amino-2-methyl-7-octynoic acid, and onchidin B, the β-hydroxy acid, 3-hydroxy-2-methyl-7-octynoic acid. The onchidins are known to be cytotoxic, but no details were given in regard to this activity (366,367). The chemical makeup suggests that the compounds originate from cyanobacteria and that they are transmitted *via* herbivorous mollusks to *Philinopsis speciosa* (368,369). Combined extracts [(EtOH and $CHCl_3$/MeOH (1:1)] of the mollusk *P. speciosa* yielded kulolide-1 (**302**), kulolide-2, kulolide-3,



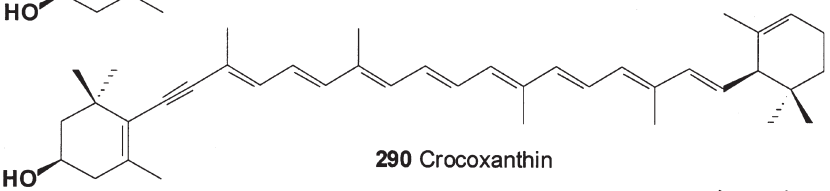
286 Crassostreaxanthin A

287 Crassostreaxanthin B

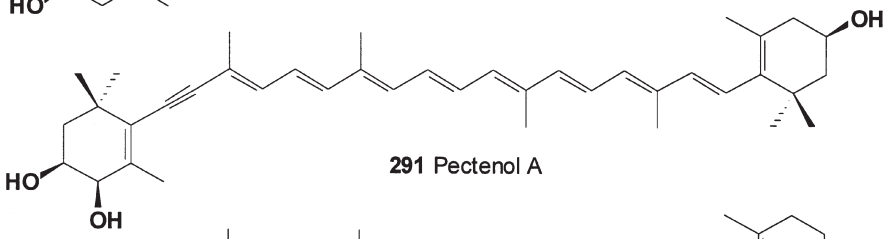
288 Apoalloxanthinal



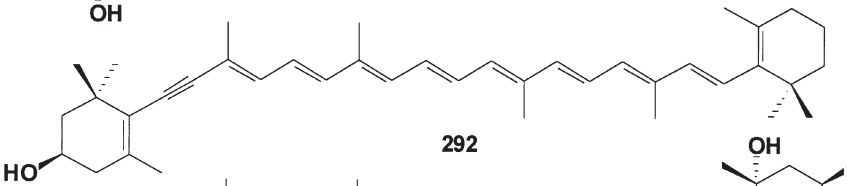
289 Alloxanthin



290 Crocoxanthin



291 Pectenol A



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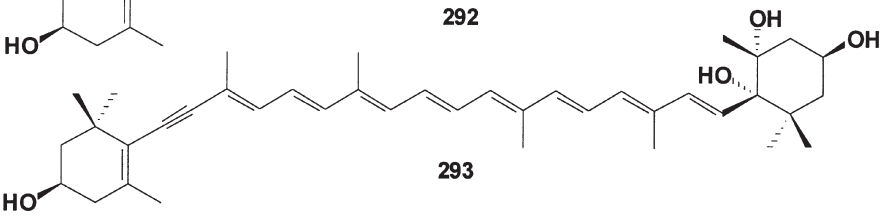

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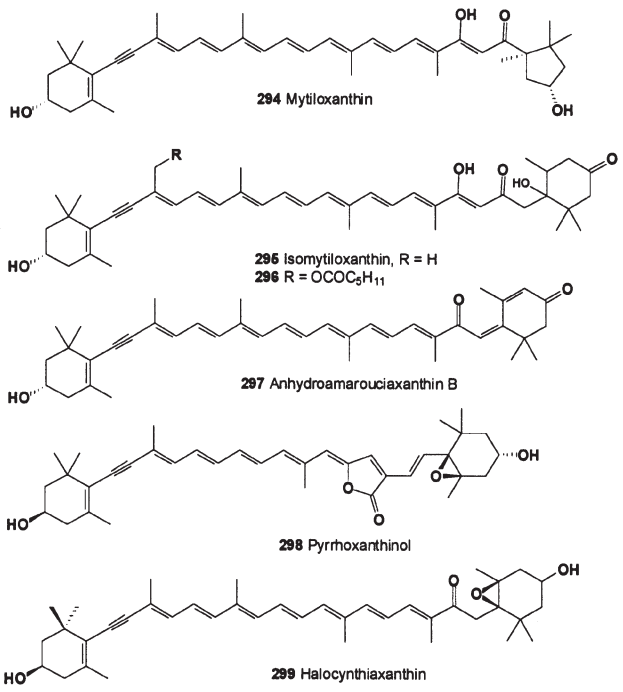
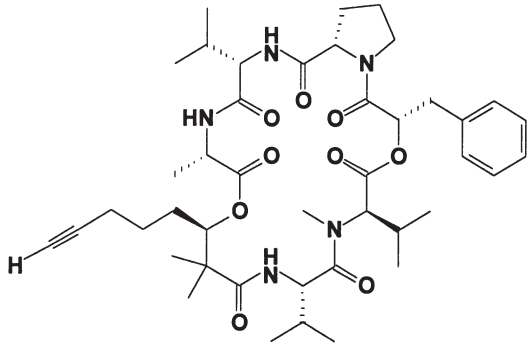
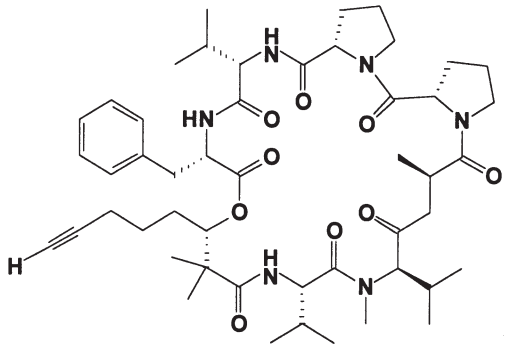
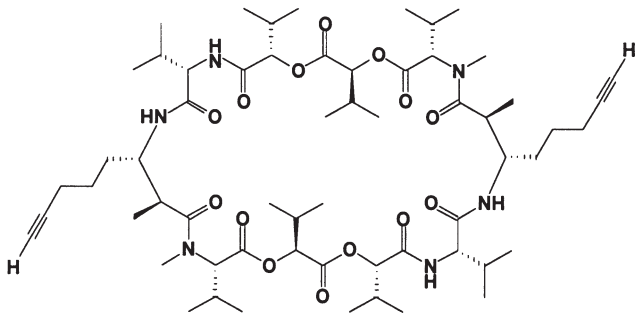
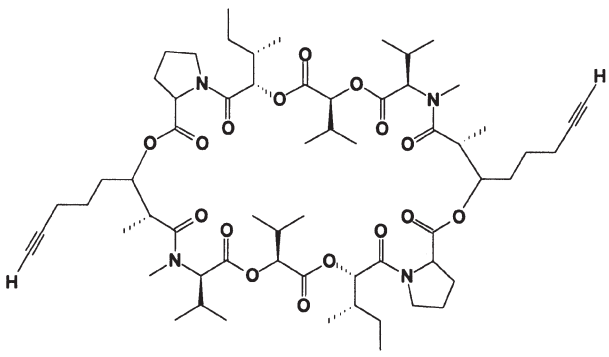








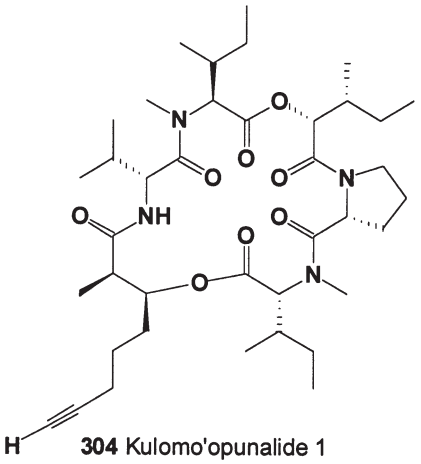


kulokainalide-1 (**303**), kulomo'opunalide-1 (**304**), kulomo'opunalide-2 (**305**), and tolytoxin 23-acetate.

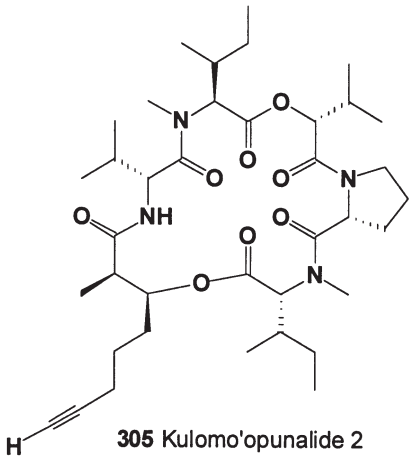
Kulolide (**302**), a cyclic depsipeptide, was isolated from a cephalaspidean mollusk, *Philinopsis speciosa* (368,369). Kulolide is made up of five amino acid residues, one each of L-Ala, L-Pro, and N-Me-L-Val and two of L-Val, and two carboxylic acids, L-3-phenylacetic acid and the unprecedented (*R*)-3-hydroxy-2,2-dimethyl-7-octynoic acid. Kulolide was active against L-1210 leukemia cells and P388 murine leukemia cells, with IC_{50} values of 0.7 and 2.1 μg/mL, respectively. Kulolide caused morphological change of rat 3Y1 fibroblast cells at a concentration of 50 μM. In addition to five new depsipeptides related to kulolide-1 (**302**), further examination of the mollusk *Philinopsis speciosa* has yielded a linear peptide, pupukeamide, and an unprecedented macrolide, tolytoxin-23-acetate.

Kulolide-1 (**302**) caused morphological changes to rat fibroblast cells at a concentration of 50 μM. Less than 0.1% contamination with tolytoxin might account for this activity. Peptides **303–305** showed only moderate cytotoxicity against P388 cells.

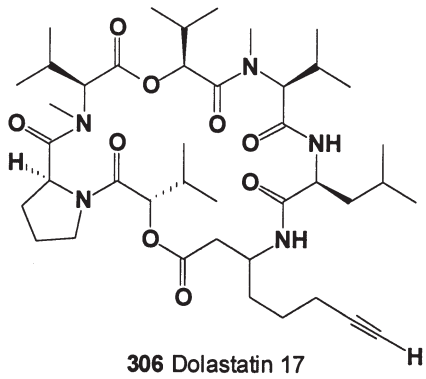
The unusual cyclodepsipeptide dolastatin 17 (**306**) was isolated from the Papua New Guinea sea hare *Dolabella auricularia* (Gastropoda, Orthogastropoda, Aplysiidae) and found to contain an acetylenic β-amino acid, designated dolayne. Dolastatin 17 exhibited significant human cancer cell growth inhibitory activity (GI_{50} 0.45–0.74 μg/mL range) (370,371).



304 Kulomo'opunalide 1



305 Kulomo'opunalide 2



306 Dolastatin 17

STARFISH AND OTHER ECHINODERMS

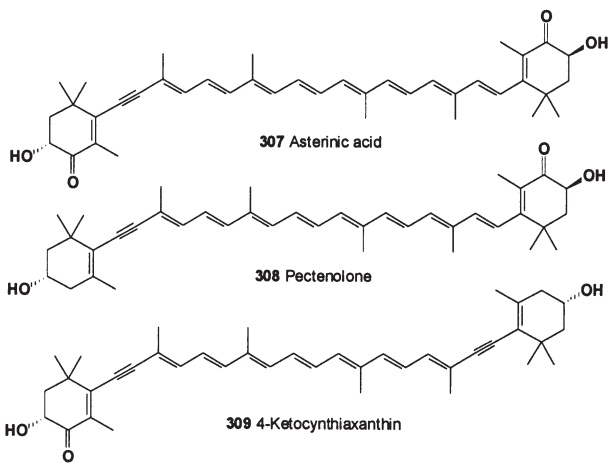
Echinoderms ("spiny skin" in Greek: including starfish, brittle stars, crinoids, sea urchins, and sea cucumbers) are radially symmetrical invertebrates that are only found in the sea (372–374). More than 6,500 species have been recorded in the phylum Echinodermata. A variety of biologically active substances have been isolated from the echinoderms: carotenoids, ether lipids, glycolipids, saponins, naphthoquinones, porphyrins, and others; some of the isolated metabolites contain the acetylenic unit (375–377). Several substances unique to echinoderms have also been reported, some of which show high potential as new medicaments.

From the calyx and arms of *Lamprometra klunzingeri* (family Mariametridae, class Crinoidea, Echinodermata), collected in the Red Sea, the cytotoxic carotenoids diadinochrome (**170**), alloxanthin (pectenoxanthin = **289**), cynthiaxanthin, and asterinic acid (**307**) have been isolated (378).

Asterinic acid (**307**) was found in the following echinoderms from the Adriatic Sea: *Coscinasterias tenuispina, Marthasterias glacialis, Paracentrotus lividus,* and *Sphaerechinus granularis* (379). The cytotoxic acetylenic carotenoids diatoxanthin, and alloxanthin were present in the gonads of Australian and Japanese species of the echinoids *Heliocidaris erythrogramma* and *H. tuberculata* (380); in the sea urchin *Pseudocentrotus depressus* (381); in the sea urchin *Peronella japonica* (382); in seven species of sea urchins, belonging to the orders Cidaroida, Echinothurioida, Diadematoida, and Arbacioida, as well as pectenolone (**308**) and 4-keto-cynthiaxanthin (**309**) (383).

Asterinic acid was isolated from *Asterias rubens*, *Acanthaster planci*, *Coscinastrias acutispina*, *Leiaster leachii*, *Asterias amurensis*, *Ophidiaster ophidianus*, *Asterina panceri*, *Asteropecten aurantiacus*, and *Marthasterias glacialis* (384–389). Mytiloxanthin was found in *Ophiocomina nigra* (390); and derivatives of the cytotoxic acetylenic carotenoids, (3*S*,4*S*,3′*S*,5′*R*)-4-hydroxymytiloxanthin, (3*S*,4*S*,3′*S*,4′*S*)-4,4′-dihydroxydiatoxanthin, (3*S*, 4*S*,3′*S*,4′*S*)-4,4′-dihydroxyalloxanthin, (3*S*,3′*S*,4′*S*)-4-keto-4′-hydroxydiatoxanthin, and (3*S*,3′*S*,4′*S*)-4-keto-4′-hydroxyalloxanthine were isolated from the starfish *Asterina pectinifera* and *Asterias amurensis* (391).

Carotenoid content of the seven species of sea cucumber (*Stichopus japonicus*, *Holothuria leucospilota*, *H. moebi*, and *H. pervicax* of the order Aspidochirotida, *Cucumaria japonica*, *C. echinata*, and *Pentacta australis* of the order Dendrochirotida) was reported (392). β-Carotene, β-echinenone, canthaxanthin, phoenicoxanthin, and astaxanthin were common in all the sea cucumbers examined. Alloxanthin, diatoxanthin, and pectenolone were isolated as minor carotenoids. The bluish vi-



307 Asterinic acid

308 Pectenolone

309 4-Ketocynthiaxanthin

olet pigment in the dorsal skin of *Asterias rubens* was isolated as an amorphous powder (393). Asterinic acid (**307**) and four derivatives of alloxanthin, diatoxanthin, and mytiloxanthin were isolated and their structures elucidated. More detailed information on the carotenoids and other bioactive compounds was also reported (394,395).

TABLE 20
Cytotoxicity[a] of Namenamicin and Shishijimicins

Cell line	315	316	317	318
2Y1	13.0	2.0	3.1	4.8
HeLa	34.0	1.8	3.3	6.3
P388	3.3	0.47	2.0	1.7

[a]IC_{50}, pg/mL. Reference 402. For abbreviation see Table 2.

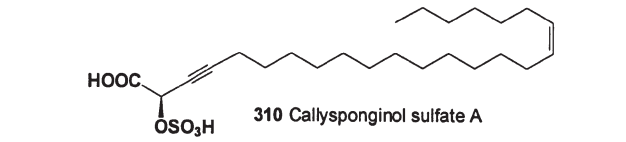
TUNICATES (OR SEA SQUIRTS)

Tunicates (urochordates) are small, box-like filter-feeding animals that live either alone or in colonies cemented to the sea floor. Many species of tunicata produce bioactive compounds (396). Callysponginol sulfate A (**310**), a sulfated C_{24} acetylenic fatty acid from the marine sponge *Callyspongia truncata*, is a membrane type 1 matrix metalloproteinase (MT1-MMP) inhibitor. Compound **310** inhibited MT1-MMP with an IC_{50} value of 15.0 mg/mL (397), and sodium 1-(12-hydroxy)octadecanyl sulfate was isolated from a marine tunicate as a matrix metalloproteinase 2 (MMP2) inhibitor (398). This compound inhibited MMP2 with an IC_{50} value of 9.0 μg/mL.

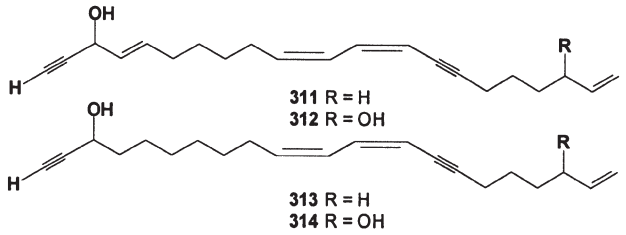
Four cytotoxic straight-chain polyacetylenic alcohols (**311–314**) were isolated from a marine ascidian (phyllum Chordata, subphyllum Urochordata) collected off Vigo, along the Atlantic coast of northwestern Spain (399). This is the first finding of acetylenic lipids from an organism belonging to phylum Chordata.

The carotenoids of the muscles and tunic of the tunicates *Halocynthia aurantium*, *H. roretzi, Styela clava*, and *S. plicata* were isolated and identified (400,401). Antimutagenic activities of the carotenoids toward *S. typhimurium* TA 98 and cytotoxic activity for cancer cell lines were detected. Total carotenoid contents in the muscle of tunicata ranged from 18.65 to 2.39 mg percent. The major carotenoids of *H. roretzi*, *H. aurantium*, *S. plicata*, and *S. clava* were cynthiaxanthin (25.1–42.2%), halocynthiaxanthin (9.7–26.3%), diatoxanthin (8.0–18.7%), and β-carotene (7.7–21.7%). Diadinochrome, mytiloxanthin, diatoxanthin, alloxanthin, pectenolone, and halocynthiaxanthin were isolated from *H. roretzi* (400,401). Halocynthiaxanthin (**299**), with few side effects at 2 μg/mL, inhibited growth of HeLa, COLO32ODM, HGC-27, PANC-I, and GOTO cells, *in vitro* (402). Halocyanthiaxanthin (5 μg/mL), from the sea squirt *H. roretzi*, caused complete suppression of human neuroblastoma GOTO cell proliferation, reducing the growth rate by 88.8% compared with the control. Furthermore, halocyanthiaxanthin also inhibited the growth of other human malignant tumor cells. Thus, halocyanthiaxanthin seems to be a promising antineoplastic agent (403).

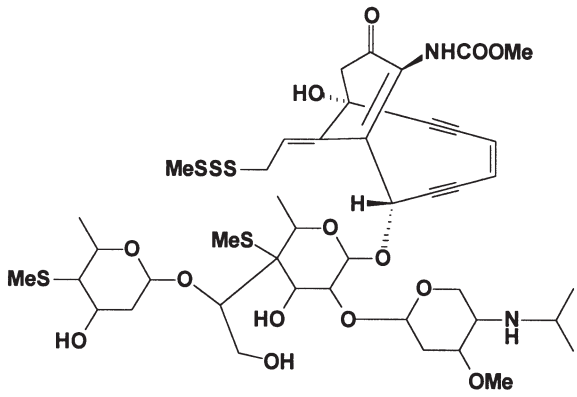
Extracts of the colonial marine ascidian, *Polysyncraton lithostrotum*, collected from Namenalala Island, Fiji Islands,



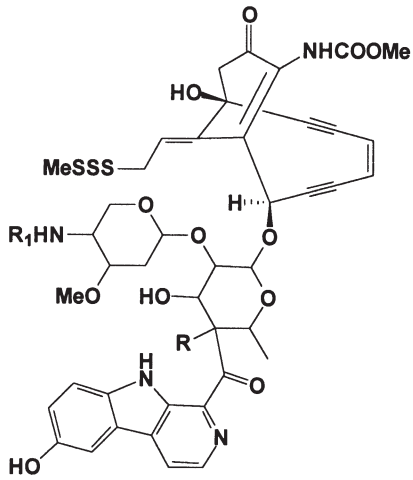
310 Callysponginol sulfate A



311 R = H
312 R = OH
313 R = H
314 R = OH



315 Namenamicin



316 Shishijimicin A, R = SMe, R_1 = iPr
317 Shishijimicin B, R = H, R_1 = iPr
318 Shishijimicins C, R = SMe, R_1 = Et

showed induction of the SOS repair response in a Biochemical Induction Assay (BIA) and potent cytotoxicity against a panel of human tumor cell lines. Bioassay-guided fractionation of the extract, following BIA activity, yielded namenamicin (**315**), a new enediyne antitumor antibiotic. DNA cleavage experiments showed that namenamicin cleaves DNA less specifically than calicheamicin (404). The MeOH and EtOH extracts of the tunicate, following reversed-phase HPLC, afforded shishijimicins A (**316**), B (**317**), and C (**318**), together with the known namenamicin (Table 20).

CONCLUDING REMARKS

Intensive searches for new classes of pharmacologically potent agents produced by bacteria, cyanobacteria, micro- and macroalgae, marine and freshwater invertebrates, plants, and fungi have resulted in the discovery of dozens of compounds possessing high cytotoxic activities. However, the preclinical and clinical development of many terrestrial- and aquatic-derived natural products into pharmaceuticals is often hampered by a limited supply from the natural source. Total synthesis is of vital importance in these situations, allowing for the production of useful quantities of the target compound for further biological evaluation.

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Polyunsaturated Phosphatidylinositol and Diacylglycerol Substantially Modify the Fluidity and Polymorphism of Biomembranes: A Solid-State Deuterium NMR Study

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ABSTRACT: One of the main biological systems that can be used as a model for studying the molecular mechanisms involved in membrane fusion is the formation of the nuclear envelope (NE). NE assembly to form the male pronucleus at fertilization occurs by binding of NE membrane precursor vesicles to chromatin and their fusion. MV_1 is an NE precursor vesicle population of low density, highly enriched in {18:0/20:4}PI. The modification of {18:0/20:4}PI to {18:0/20:4}DAG leads to NE formation, and the depletion of MV_1 from the total membrane precursors results in the inhibition of NE assembly. Here we show by ^{2}H NMR studies of various physiologically relevant model membranes made of {18:0/20:4}PI, {18:0/20:4}DAG, and saturated and unsaturated PC that membranes of composition similar to MV_1 exhibit dramatically enhanced fluidity and non-lamellar structures, thus providing a possible explanation for the essential role of MV_1 and the modification of PI to DAG in membrane precursor vesicles during NE assembly.

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The nuclear envelope (NE) is disassembled and reassembled at each mitosis in typical animal cells. Membrane vesicle precursors of different phospholipid compositions contribute to the membrane fusion leading to NE formation (1). Therefore, study of NE assembly combines and relates the study of membrane domains and regulation of membrane fusion.

Study of male pronuclear membrane formation in fertilized sea urchin egg extracts has revealed several novel features of assembly, especially regarding the role of phospholipids. The binding of NE vesicles to chromatin is ATP-dependent (1). When total precursor membrane vesicles are fractionated on a sucrose gradient, several vesicle populations can be resolved (2). The least dense, MV_1, is highly enriched in PI (3,4). This unusual precursor membrane vesicle plays an essential role in the assembly of the NE. *In vitro*, assembly can be triggered by either GTP hydrolysis or bacterial or eukaryotic phosphoinositide phospholipase C (PIPLC), and depletion of MV_1 from the cell-free system prevents NE assembly (4,5).

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Abbreviations: DPPC, diplamitoylphosphatidylcholine; NE, nuclear envelope; PIPLC, phosphoinositide phospholipase C; POPC, palmitoyloleoylphosphatidylcholine.

Fusion of membrane vesicles is a proteo-lipid phenomenon in which proteins are required for targeting and fusogenic lipids for destabilizing the local structure of the lamellar bilayer to induce fusion. Model membrane studies have shown that lipids such as DAG, PE, and PA are destabilizing fusogenic lipids, and that variation in their composition induces membrane fusion (6–9). Our EI–MS studies show that the major PI in the total membrane vesicle population is {18:0/20:4}PI, and its hydrolysis by bacterial PLC leads to production of {18:0/20:4}DAG (10). Moreover, we have recently shown that DAG derived from $PI(4,5)P_2$ hydrolysis is required to induce NE assembly (5).

Studies performed on the physical properties of membranes have been based largely on model systems, but these do not necessarily represent the composition of biological membranes (11). We have exploited deuterium solid-state NMR to study membrane fluidity and polymorphism of model systems that have similar composition to the NE precursor membrane vesicles. ^{2}H NMR is one of the most precise and direct methods to determine order parameters that depict the amplitude of intramolecular and molecular motions; the method therefore provides insight into the rigidity or fluidity of a particular membrane. These physical parameters are directly associated with the fusogenity and the mobility of membranes. Thus, the order parameters of biologically relevant model membranes can be informative as to their physiological roles.

A study concerning the physical properties of biologically relevant model membranes investigated the effect of calcium ions on phase separation in natural PC and PI liposomes (12). This study primarily used ESR methods. The effect of DAG on phase changes of phospholipid bilayers has been studied using x-ray diffraction techniques (13). However, in the latter study, {18:0/20:4}DAG, which is specifically relevant to NE assembly, was not used. Moreover, neither of these studies exploited deuterium-labeled solid-state NMR to study the physical properties of high levels of natural PI and natural DAG.

The specific role of large amounts of PI in membrane conformational changes has not been previously investigated by a non-invasive method such as solid-state NMR. Solution NMR studies have illustrated that MV_1 is composed of 80 mol % PI and 20 mol % PC (4). To investigate the physical properties of such an unusual bilayer, and the consequences of hydrolysis of the PI by PIPLC, we made various binary systems with

{18:0/20:4}PI/diplamitoylphosphatidylcholine (DPPC)-$^2H_{62}$ and {18:0/20:4}DAG/DPPC-$^2H_{62}$. Because DPPC has an elevated gel-to-fluid phase transition temperature, T_c, near 41°C, we also used deuterium-labeled palmitoyloleoylphosphatidylcholine (POPC), which has a T_c near –5°C. POPC has a 16:0/18:1 chain composition that is more representative of MV_1 PC composition.

We show that with increasing molar ratios of {18:0/20:4} DAG and {18:0/20:4}PI in DAG/DPPC and PI/DPPC binary model membranes, membrane fluidity is enhanced dramatically and non-lamellar structures are promoted. Similar results are obtained using POPC containing 80% DAG or PI. We propose a plausible explanation for the presence of PI domains to account for fusion events in NE assembly.

EXPERIMENTAL PROCEDURES

Lipids were purchased from Larodan Fine Chemicals (Sweden): D1-stearin-2 arachidonin ({18:0/20:4}DAG) and PI (liver, porcine; mainly composed of {18:0/20:4}). Note that the FA molecular composition of our binary model membranes is the same as the molecular composition of the NE membrane vesicles. Deuterium-labeled 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC-$^2H_{62}$) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC-$^2H_{31}$) were purchased from Avanti Polar Lipids, Inc. (Alabama, U.S.A.). All solvents were purchased from Aldrich (Saint Quentin Fallavier, France), and 4-mm (50-µL) ZrO_2 rotors were purchased from Cortec (Paris, France).

Preparation of Multi-lamellar Vesicles. DPPC-$^2H_{62}$/PI, DPPC-$^2H_{62}$/DAG, POPC-$^2H_{31}$/PI, and POPC-$^2H_{31}$/DAG mixtures in chloroform were mixed by vortexing and dried down in a Speedvac for 20 min. The 1:4, 1:1, and 4:1 molar ratios were made for binary model membranes with DPPC. Only the 4:1 molar ratio was made when preparing binary mixtures with POPC. Pellets were resuspended in 200 µL of TN buffer (10 mM Tris-HCl, 150 mM NaCl, 15 mM $MgCl_2$, pH 7.2) and lyophilized. Lipid powders were dispersed in deuterium-depleted water at the required concentration to give 80% hydration (wt/wt). Hydration was completed by three 20-min freeze-thaw cycles. The samples were placed in 50-µL ZrO_2 rotors. Depending on samples, the amount of deuterated lipid ranged from 3 to 12 mg.

NMR Spectroscopy. Solid-state NMR was carried out on Bruker Avance DSX-500 and DSX-300 instruments (11.75 and 7.05 Tesla, respectively). ^{31}P NMR spectra were acquired at 202 MHz using a phase-cycled Hahn-echo pulse sequence with gated broadband proton decoupling (14). ^2H NMR experiments on deuterated lipids were performed at 46.1 MHz by means of a quadrupolar echo pulse sequence (15). Typical acquisition parameters were as follows: spectral window of 50 kHz for ^{31}P NMR and 250 kHz for ^2H NMR; π/2 pulse widths ranged from 3 to 5 µs depending on sample, interpulse delays were of 30–40 µs. A recycle delay of 5 s was used for ^{31}P NMR; for ^2H NMR experiments, it was set to 2 s. Typically, 2k–4k scans were recorded for the deuterium nucleus and 4k transients for the phosphorus nucleus. A line broadening of 50–300 Hz was applied prior to Fourier transformation. Phosphorus chemical shifts were referenced relative to 85% H_3PO_4 (0 ppm). Quadrature detection and digital filtering were used in all cases. Samples were allowed to equilibrate at least 30 min at a given temperature before the NMR signal was acquired; the temperature was regulated to ±1°C.

Calculations. The first spectral moment, M_1, was calculated according to the method of Davis (16) using a C++ routine analysis incorporated in Origin 7 software (S. Buchoux, CNRS, University of Bordeaux 1, IECB, Bordeaux, France, unpublished). To avoid zero values for spectra symmetrical relative to the origin a sign reversal was used for negative frequencies. In such a case M_1 can be used to directly estimate the carbon-deuterium bond order parameter S_{CD} according to the following equation:

$$\langle S_{CD} \rangle = \frac{\sqrt{3}}{\pi A_Q} M_1 \qquad [3]$$

Angular brackets represent the average overall deuterium-labeled positions in the perdeuterated palmitic chains in DPPC and POPC. A_Q is the static quadrupolar coupling constant, 167 kHz (17).

RESULTS

For the initial optimization of our studies we prepared model systems with molar ratios of PI or DAG in DPPC of 1:4, 1:1, and 4:1. Because pure DPPC membranes have a gel-to-fluid transition temperature around 40°C, we performed temperature scans from 0 to 60°C, by increasing or decreasing temperatures. Reproducible spectra were obtained independent of the thermal history of the sample. To investigate a more physiologically relevant model system we used POPC and applied the same procedures as with DPPC. In the case of POPC, only the 1:4 molar ratio between POPC and DAG or PI was prepared. We performed temperature scans from –15°C to 40°C, because the POPC gel-to fluid phase transition temperature occurs at about –5°C. Similar to DPPC-containing samples, the spectra obtained were independent of the thermal history of the sample.

Figure 1 shows selected ^2H NMR spectra in the presence of PI. The left panel shows control DPPC-$^2H_{62}$ spectra. A well-known axially asymmetric L_β, gel phase spectrum is detected at 20°C depicting the quasi-absence of intramolecular motions and a molecular tilt of approximately 30° with respect to the membrane normal (16). At 60°C a narrower axially symmetric spectrum is detected, representative of intramolecular disorder (gauche-trans isomerizations of chain segments, whole molecule wobbling) as in an L_α fluid phase. At 35°C, a two-phase spectrum is detected, illustrating the coexistence of gel and fluid phases on the NMR timescale (µs). Addition of PI induces, overall, a narrowing of all spectra. This is particularly clear for the sample containing 80% PI, for which the spectrum at 20°C is characteristic of a fluid phase. This narrowing phenomenon is also observed at high temperatures. For various amounts of PI, a more general view can be obtained when plot-

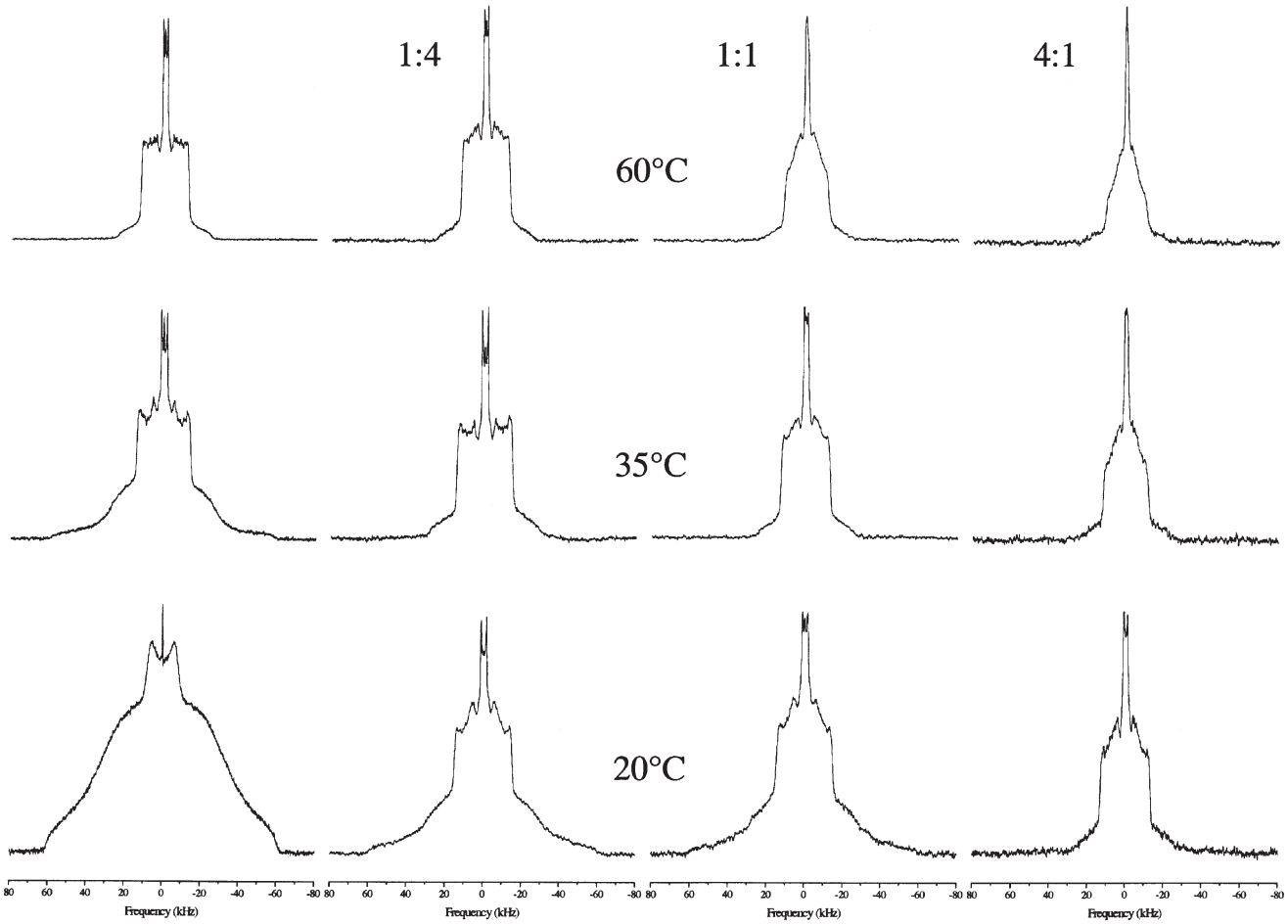

FIG. 1. Elevated amounts of PI in PI:DPPC induce membrane fluidity. Representative deuterium wide-line NMR spectra of DPPC-$^2H_{62}$ in the absence (left panel) or presence of PI. Temperatures and {18:0/20:4}PI:DPPC molar ratios are indicated. Sample hydration is 80%. Depending on DPPC amounts (3–12mg), each spectrum is the result of 1k to 4k scans. A Lorentzian filtering (LB) of 200 Hz was applied prior to Fourier transformation.

ting the first moment as a function of temperature (Fig. 2). Several effects are noted: reduction of molecular order at low temperatures, downshift of the gel-to-fluid phase transition, and molecular disorder in the fluid phase spectra. A clear dose-dependent effect is demonstrated.

We have translated M_1 into membrane order by plotting on a double-*Y* axis the orientation order parameter S_{CD} (Fig. 2). A value close to 1 represents membranes where almost no bond or molecule fluctuations are detected (ordered system). A zero value shows complete molecular disorder as in liquids. It is striking that elevated amounts of PI induce membrane disorder in a manner that the membrane at 15°C with 80% PI has the same fluidity as pure DPPC above its fluid phase transition temperature (40°C).

Figures 3 and 4 show the corresponding effect of DAG on DPPC-$^2H_{62}$ liposomes. Spectral features become narrower as the amounts of DAG are increased (Fig. 3). At high temperatures an isotropic line dominates the spectrum, indicating that the initial micrometer-size multi-lamellar vesicles have been converted into isotropic symmetries such as cubic phases or smaller tumbling objects (50–200 nm) (18,19). This is a clear indication that DAG modulates the vesicle size by inducing a highly curved interface. From the M_1-vs.-*T* graph (Fig. 4) we can see that at low temperatures, where values typical of a gel phase are detected, reduced membrane perturbations occur. A 10°C downshift of the gel-to-fluid phase transition temperature is detected, and there seems to be a saturation effect at 20 mol % DAG in DPPC. DAG readily promotes membrane disorder for amounts greater than 20 mol % and temperatures higher than 40°C. It should be noted here that the presence of the isotropic line for very high temperatures leads to an additional decrease of M_1 that cannot be attributed to disorder.

Figure 5 shows selected spectra of POPC-$^2H_{31}$ in the absence or presence of 80 mol % DAG (center) or PI (right). The features observed for pure DPPC in Figure 1 are also observed with POPC, but these are downshifted in temperature by approximately 45°C. Below –5°C, gel-phase spectra are detected; above this temperature, axially symmetric powder patterns characteristic of lamellar fluid phases are observed. This downshift in temperature is well known and is due to the presence of

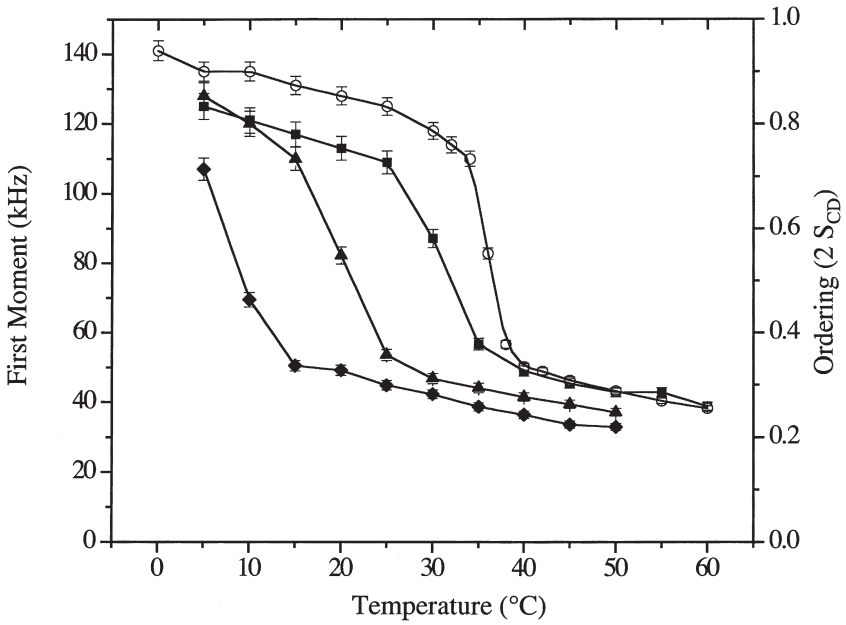

FIG. 2. Thermal variation of the first spectral moment of PI:DPPC membranes. Calculated from ^{2}H NMR spectra of DPPC-$^2H_{62}$ in the absence (empty circles) or presence of {18:0/20:4}PI; {18:0/20:4}PI:DPPC molar ratios of 1:4 (squares), 1:1 (triangles), and 4:1 (diamonds). Accuracy of calculations is ±3%. On the double *Y*-axis the C-D order parameter is shown as calculated from Equation [1] (see text). Twice $<S_{CD}>$ is plotted to express residual ordering information relative to the bilayer normal. A zero value is indicative of a fully disordered system, as in pure liquids, whereas a value of 1 reflects the absence of *gauche-trans* isomerization and molecular wobbling as in a very rigid membrane (ordered system).

the *cis* double bond at positions 9–10 in the *sn*-2 chain that hinders the close packing of acyl chains. In the presence of DAG (center panel), we note the appearance of a marked isotropic line that dominates spectra at high temperatures. With PI (right panel), the spectrum at –10°C is in the fluid phase and remains characteristic of this phase for increasing temperatures. In fact, to detect an ordered system the temperature had to be decreased to –15°C (data not shown). At 40°C, the spectrum in the presence of PI is much narrower that that of pure POPC, clearly indicating a greater disorder of the membrane. It is noteworthy that the deuterium-labeled lipid shows only one spectrum in the mixed system (independent of the PC/(PI or DAG) ratio). The spectrum is different from pure PC, indicating that the deuterated reporter lipid samples only one environment on the microsecond timescale of NMR. This shows that there is only one average homogeneous environment. A sample with coexisting rigid and fluid domains would have shown two spectra, one of them being the pure reporter (POPC or DPPC). This phenomenon is clearly not observed. In addition, the deconvolution of deuterium spectra was performed using the de-Pakeing algorithm (20), and the order parameter profile was plotted against the labeled carbon position. This shows that all chain positions, from the glycerol backbone near the interface to the bilayer center, are disordered in a similar way (data not shown).

In order to ascertain the effects detected by the chains of labeled POPC, we performed ^{31}P NMR experiments on new samples of unlabeled POPC with 80 mol % {18:0/20:4}PI or {18:0/20:4}DAG. Figure 6 shows that with high levels of DAG, the 40°C spectrum is characterized by a single isotropic line. This profile is also as observed with ^{2}H NMR (Fig. 5). However, at –5°C a broad feature is barely detected. The weak intensity is due to the low amount of lipids containing phosphorus (20% POPC only) that show a spectral feature with a wide distribution of frequencies, as also observed with ^{2}H NMR. For the PI:POPC model system, a spectrum typical of a fluid membrane is seen both at 40 and –5°C, as also detected with ^{2}H NMR (Fig. 5). Closer analysis of the spectra illustrates that although there are similar dynamics, the two lipids show two detectable chemical shift anisotropies that reflect the different nature of the lipid head groups (PI and PC).

The data in Figure 5 were used to calculate the first spectral moment, M_1. From the M_1-vs.-T graph in Figure 7, we observe that both DAG and PI with POPC produce effects similar to DPPC, except the temperatures are downshifted by approximately 45°C. PI greatly decreases the gel-to-fluid transition temperature of POPC and increases molecular disorder of the fluid-phase spectra. DAG also shifts the transition temperature, but to a lower extent. It readily promotes membrane disorder for temperatures higher than 0°C. As with DPPC-containing samples, it should be noted that the presence of the isotropic line for very high temperatures leads to an additional decrease of M_1 that cannot be attributed to disorder.

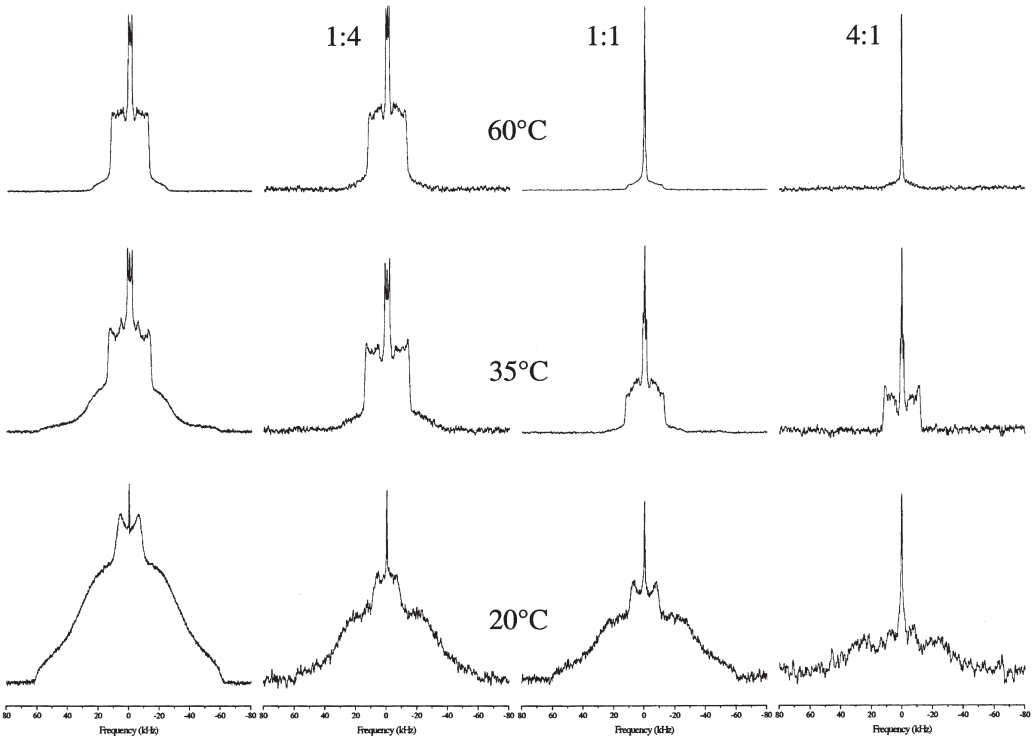

FIG. 3. Elevated quantities of DAG induce membrane disorder. Representative deuterium wide-line NMR spectra of DPPC-$^2H_{62}$ in the absence (left panel) or presence of DAG. Temperatures and {18:0/20:4}DAG :DPPC molar ratios are indicated. Sample hydration is 80%. Experimental parameters are as in Figure 1.

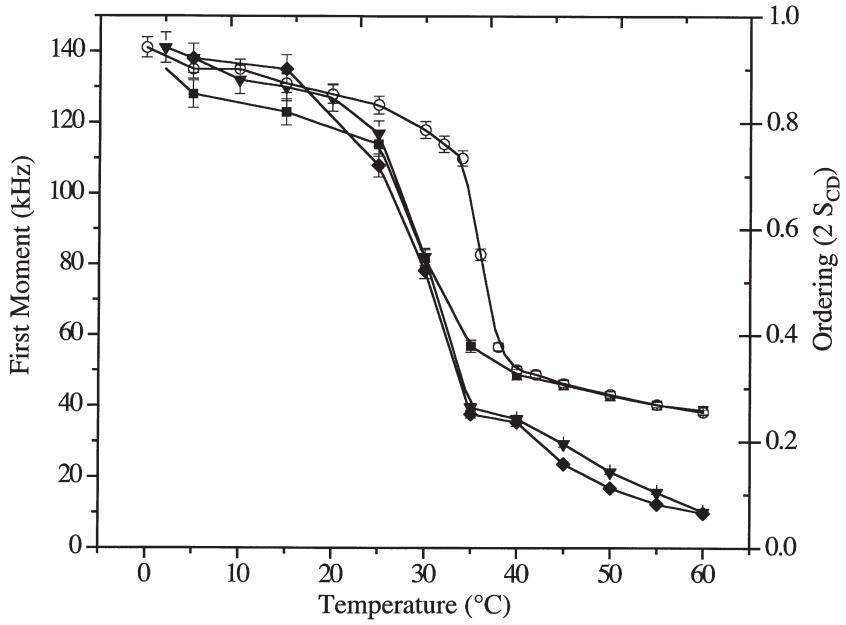

FIG. 4. Thermal variation of the first spectral moment of DAG:DPPC membranes, as calculated from ^2H NMR spectra of DPPC-$^2H_{62}$ in the absence (empty circles) or presence of {18:0/20:4}DAG; {18:0/20:4}DAG:DPPC molar ratios of 1:4 (squares), 1:1 (triangles), and 4:1 (diamonds). Other parameters are as in Figure 2.

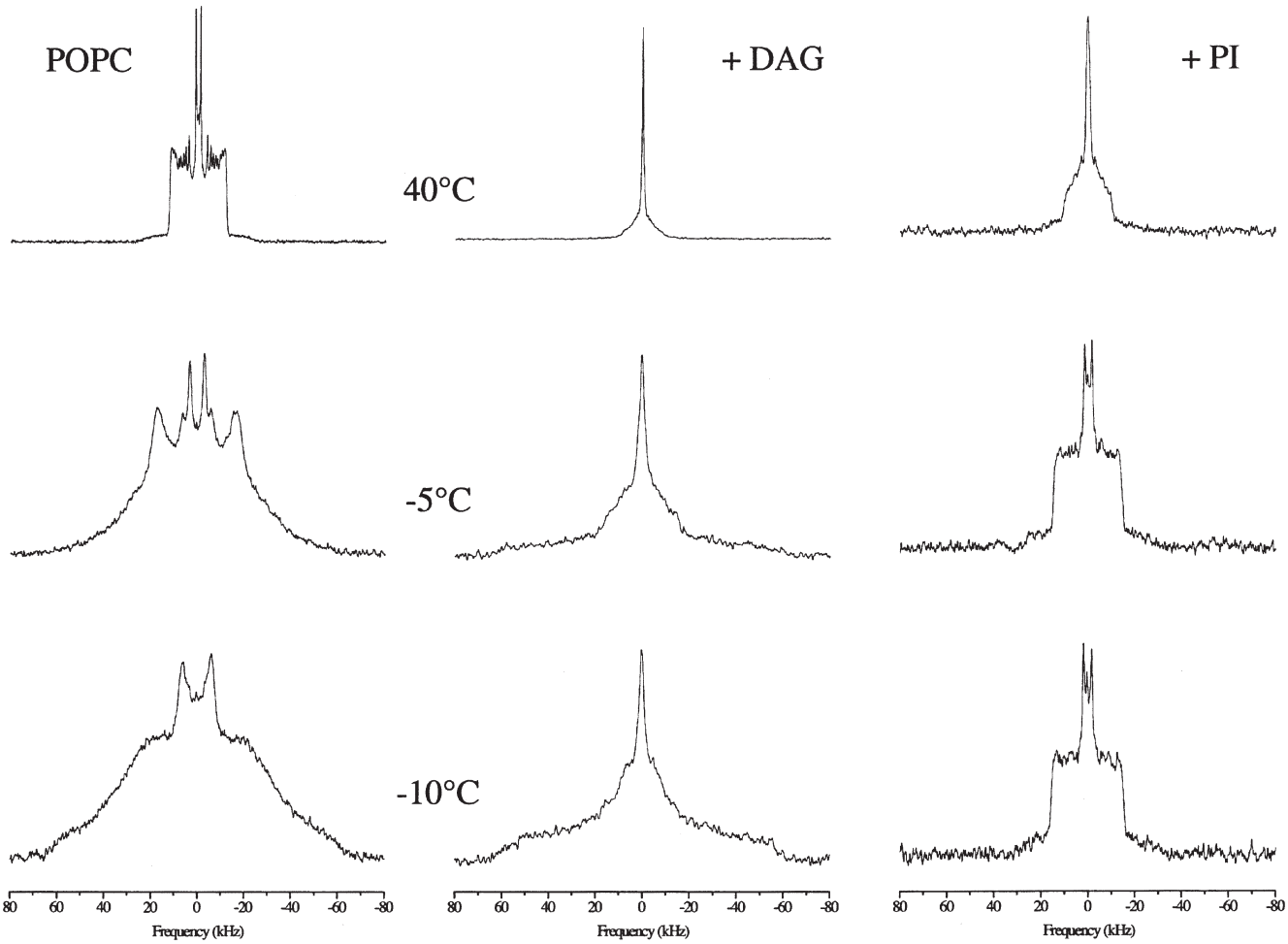

FIG. 5. Membrane perturbations with high levels of PI/POPC-$^2H_{31}$ and DAG/POPC-$^2H_{31}$. Representative deuterium wide-line NMR spectra of POPC-$^2H_{31}$ in the absence (left panel) or presence of 80 mol % DAG or PI. Temperatures are indicated on spectra. Sample hydration is 80%. Experimental parameters are as in Figure 1.

DISCUSSION

We have demonstrated by ^2H NMR studies that both polyunsaturated PI and DAG cause prominent perturbations of membrane fluidity and thermal behavior, both in saturated and unsaturated model membranes. To our knowledge, prior investigations using ^2H solid-state NMR illustrating perturbations in membrane fluidity and membrane disorder induced by high levels of {18:0/20:4}PI and {18:0/20:4}DAG lipids have not been reported. Moreover, we show that PI readily disorders membrane packing by providing fluid-phase conditions at temperatures as low as 15°C with DPPC or –10°C with POPC (sample with 80% PI). This is an important point because the physiological temperature of the sea urchin is close to 16°C. It should be noted that, typically when phase transitions are studied, experiments are performed at much higher temperatures that are not strictly physiologically relevant to biological systems. MV_1 FA composition is mainly {18:0/20:4}PI and {18:1/20:4}PC. Moreover, {18:0/20:4}PI is the main molecular species that undergoes lipid modification in our system (B. Larijani, unpublished data). Hence the study of this particular species became our prime interest. Nevertheless, the closest deuterated probes available commercially to perform our experiments were 16:0/16:0PC and 16:0/18:1PC.

We therefore used the closest binary composition to MV_1 ({18:0/20:4}PI/DPPC and{18:0/20:4}PI/POPC, 4:1 molar ratio) to study the physical properties of these systems. It should be noted that the initial experiments were performed with DPPC, but the physiological significance of the data relies on the experiments that were performed with POPC. We deduce from our data that these biological vesicles are very fluid even at low temperatures. PI provides a greater chain disorder of the membrane, which may result in a reduction of the membrane thickness (21,22). This effect could be promoted both by the PI head group and chains. It is however well documented that the augmentation of unsaturated bonds induces a greater disorder in lipid bilayers (23). We suggest that the role of this particular polyunsaturated PI is to promote such an effect, and it would be well-adapted for fusion events that require a dynamic membrane interior.

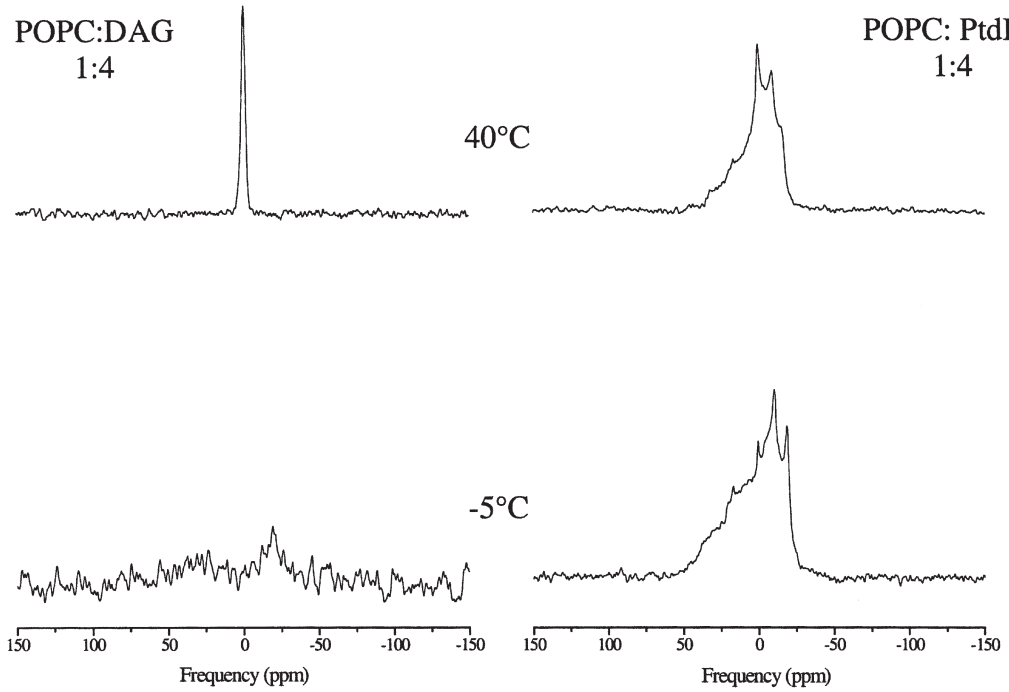

FIG. 6. Membrane perturbations with high levels of PI/POPC and DAG/POPC. Representative ^{31}P wide-line NMR spectra of POPC in the presence of 80 mol % DAG or PI. Temperatures are indicated on the spectra. Sample hydration is 80%. Each spectrum is the result of 4k scans. A Lorentzian filtering (LB) of 100–300 Hz was applied prior to Fourier transformation.

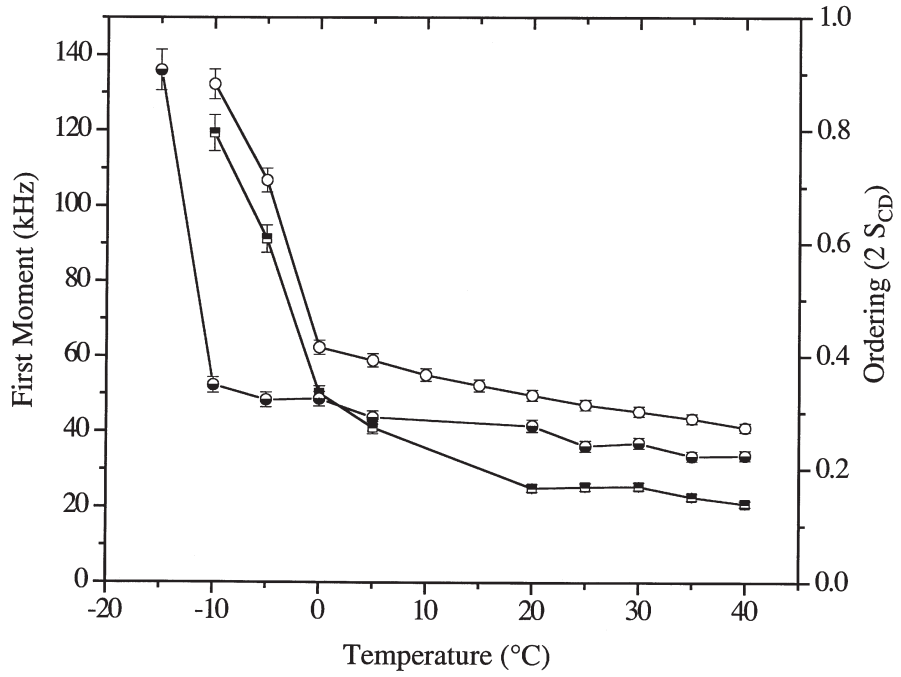

FIG. 7. Thermal variation of the first spectral moment of DAG:POPC-$^2H_{31}$ and PI:POPC-$^2H_{31}$ membranes (4:1 molar ratio), as calculated from ^2H NMR spectra of POPC-$^2H_{31}$ in the absence (empty circles) or presence of {18:0/20:4}DAG (half-filled squares) or {18:0/20:4}PI (half-filled circles). Other parameters are as in Figure 2.

In NE precursor membrane vesicles, the product of endogenous {18:0/20:4}PI hydrolysis by bacterial PIPLC is {18:0/20:4}DAG. We show in this study that unsaturated DAG does not affect rigid gel-phase membranes. However, it does induce disorder at higher temperatures, thus promoting under such conditions non-lamellar structures that are a prerequisite for induction of membrane fusion (24,25). This effect is detected independent of the chain composition of the host membrane; it occurs equally with both saturated and unsaturated PC.

From these studies we propose a model whereby both polyunsaturated PI and DAG are implicated in membrane fusion events, leading to NE assembly, in a sequential manner. The {18:0/20:4}PI in MV_1 would increase membrane fluidity thus "prompting" the membranes for fusion, and, following PLC hydrolysis of PI, highly curved structures (non-lamellar) would be formed in the presence of {18:0/20:4}DAG, thus triggering the fusion of the NE precursor membrane vesicles. Although the kinetics of membrane fusion leading to the induction of NE formation is protein-dependent we have shown in this study that lipids such as {18:0/20:4}PI can affect membrane dynamics when presented at elevated amounts such as in MV_1.

To verify this proposed model, the change in MV_1 conformation, upon PIPLC treatment, observed by ^{2}H NMR is being currently studied in our laboratories.

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Markedly Raised Intake of Saturated and Monounsaturated Fatty Acids in Rats on a High-Fat Ketogenic Diet Does Not Inhibit Carbon Recycling of ^{13}C-α-Linolenate

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ABSTRACT: Under various dietary and physiological conditions, carbon from α-linolenic acid (ALA) is extensively recycled into saturated and monounsaturated fatty acids. In this study we investigated whether carbon is still recycled from ALA when a dietary source of saturated and monounsaturated fatty acids is provided in excess. ^{13}C-labeled ALA was given to rats consuming a high-fat ketogenic diet and to rats consuming a low-fat control diet. In rats on the ketogenic diet, ^{13}C recycling from α-linolenate into several, but not all, saturated and monounsaturated fatty acids matched or exceeded that in the controls ($P<0.05$). We conclude that carbon recycling into saturated and monounsaturated fatty acids persists when the main end products of ALA recycling are provided in excess, using a ketogenic diet.

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Studies addressing the whole-body utilization of linoleic acid (LA) and α-linolenic acid (ALA) in young rats have shown that up to 80% of dietary derived LA or ALA normally disappears from the body; it is thought to be mostly or completely β-oxidized to CO_2 (1,2). In rats, recycling of exogenously administered ^{13}C-LA or ^{13}C-ALA into *de novo* products of lipid synthesis accounts for a significant proportion of the carbon skeleton of these fatty acids that is catabolized, but not completely oxidized to CO_2 (3,4). Approximately 50–60% is oxidized to CO_2, whereas the remaining 20–30% is recycled into *de novo* lipid products (3,4). This quantitative aspect of carbon recycling has been confirmed for ALA in particular, in the fetuses and infants of monkeys that were fed an LA-enriched diet throughout gestation and lactation (5). More recently, ALA recycling has also been confirmed in adult humans (6).

ALA recycling into *de novo* lipid products appears to be a preferential pathway of ALA utilization in tissues because it occurs regardless of dietary n-3 PUFA status (7,8). In young rats, ALA recycling into *de novo* lipid products is not altered by ALA deficiency during early development, a period when the demand for n-3 PUFA is thought to be at its highest (7). Conversely, adult rats fed a diet enriched with DHA continue to recycle exogenously administered ^{14}C-ALA into sterols and products of *de novo* fatty acid synthesis (8). These studies suggest that ALA recycling into sterols and saturated and monounsaturated fatty acids may possibly be an "obligatory" pathway for ALA utilization in rats (9).

It is not known, however, whether ALA recycling into *de novo* lipid products is maintained when the major fatty acid end products of ALA recycling, specifically saturated and monounsaturated fatty acids, are provided in the diet in excess. To elucidate further the nature of ALA recycling in liver, adipose, and brain, we investigated ^{13}C-ALA recycling into saturated and monounsaturated fatty acids in rats consuming a high-fat ketogenic diet. We hypothesized that, despite higher tissue incorporation of saturated and monounsaturated fatty acids in rats on a high-fat ketogenic diet, carbon recycling into products of *de novo* fatty acid synthesis would remain a major pathway of ALA utilization. This study was part of a previous study addressing the effects of the ketogenic diet on ketone body production and tissue fatty acid composition (10).

EXPERIMENTAL PROCEDURES

Animals and treatments. Male Wistar rats (Charles River, La Prairie, QC, Canada) aged 40–42 days received a low-fat control diet during the 10-day adaptation period. For the following 10 days, the control rats continued on the low-fat control diet, whereas the experimental group received a high-fat ketogenic diet (n = 8/group). The macronutrient profile of the control diet (by weight) was 8.2% fat, 9.2% protein, and 82.6% carbohydrate, whereas that of the ketogenic diet was 72.7% fat, 16.3% protein, and 11.0% carbohydrates. The control diet contained 79.3 g added fat/1000 g of diet of the following composition: palmitate (9.4%), stearate (4.2%), oleate (27.2%), LA (50.6%) and ALA (8.0%). The ketogenic diet contained 688.8 g fat/1000 added g of diet, including medium-chain fatty acids (<12 carbons; 11.3%), myristate (7.3 %), palmitate (23.9%), stearate (10.9%), oleate (25.7 %), LA (16.2%), and ALA (2.4%), as well as behenate, palmitoleate, and vaccinate, which collectively accounted for <2.3%.

After 9 d on the respective diets, six rats from each group were gavaged with 5 mg of ^{13}C-ALA dissolved in 100 μL of

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Abbreviations: ALA, α-linolenic acid; LA, linoleic acid.

TABLE 1
Fatty Acid Content of Adipose Tissue, Liver, and Brain in Rats Fed a Control or High-Fat Ketogenic Diet

	Tissue:	Adipose		Liver		Brain	
Acid	Treatment:	Control	Ketogenic Diet	Control	Ketogenic Diet	Control	Ketogenic Diet
16:0	% of total fatty acids[1] or mg/g[2]	21.2 ± 1.0	21.4 ± 0.7	15.3 ± 3.6	22.2 ± 1.7*	7.4 ± 0.2	7.9 ± 0.5*
18:0	% of total fatty acids[1] or mg/g[2]	3.1 ± 0.3	4.4 ± 0.4*	5.2 ± 0.5	8.8 ± 0.5*	4.6 ± 0.2	4.4 ± 0.2
16:1n-7	% of total fatty acids[1] or mg/g[2]	5.3 ± 0.8	3.6 ± 0.5	2.0 ± 0.6	1.0 ± 0.2*	0.2 ± 0.01	0.2 ± 0.04
18:1n-9	% of total fatty acids[1] or mg/g[2]	28.6 ± 1.1	29.2 ± 0.8	14.3 ± 5.6	21.7 ± 2.1*	8.6 ± 0.9	9.2 ± 0.6
18:3n-3	% of total fatty acids[1] or mg/g[2]	2.1 ± 0.1	1.7 ± 0.1*	1.1 ± 0.7	2.0 ± 0.3*	ND	ND

Data are the mean ± SD of n = 7–8/group.
16:0, palmitate, 18:0, stearate; 16:1n-7, palmitoleate; 18:1n-9, oleate; 18:3n-3, α-linolenate; NA, not available; ND, not detected.
[1]% of total fatty acids for adipose tissue only [2]mg per g of tissue for liver and brain.
*$P < 0.05$.

olive oil. The remaining two rats in each group were gavaged with 100 μL of olive oil and used as references for ^{13}C background during analysis of isotopic enrichment. Twenty-four hours after tracer dosing, all rats were sacrificed by an intraperitoneal injection of 1.3 μL/kg of sodium pentobarbital (MTC Pharmaceuticals, Cambridge, ON), and liver, brain, and perirenal adipose tissue samples were excised from each rat. Liver and brain were washed with saline and weighed immediately after extraction. Samples were stored at below −20°C until they were analyzed.

Lipid extraction and fatty acid analysis. As previously described (10), total lipids of homogenized samples of adipose, liver, and brain were extracted according to Folch *et al.* (11) and analyzed by gas-liquid chromatography, using a 30-m × 0.25-mm i.d. capillary column (J&W Scientific DB-23, Folsom, CA) in a Hewlett-Packard 6890 gas liquid chromatograph (Palo Alto, CA). Methyl esters of total fatty acid extracts from adipose tissue, liver, and brain were analyzed for ^{13}C enrichment by gas chromatography–combustion–isotope ratio mass spectrometry (Europa 20-20 with Orchid combustion interface, PDZ Europa, Crewe, Cheshire, UK), as described in detail elsewhere (12). The ^{13}C enrichment data are expressed as the amount of labeled fatty acid per gram of tissue (ng ^{13}C/g of tissue).

Statistical analysis: All data are presented as mean ± standard deviation. Statistical analysis was performed on Statistical Analysis Software (version 8.02, SAS Institute, Cary, NC). An unpaired Student's *t*-test was used to detect statistical significance at $P < 0.05$.

RESULTS

There were no significant differences in liver or brain weights between the control and ketogenic diet groups (10.6 ± 1.1 g in controls vs. 10.5 ± 0.7 g in ketogenic diet group for liver; 1.9 ± 0.1 g in controls vs. 1.8 ± 0.2 g in ketogenic diet group for brain). Fatty acid profiles of adipose tissue, liver, and brain reflected the differences in fatty acid composition of the control and ketogenic diets (Table 1). Compared to rats on the control diet, rats on the ketogenic diet had significantly higher percent composition of stearate (300%) in adipose tissue; higher concentrations (mg/g of tissue) of palmitate (41%), stearate (61%), and oleate (58%) in liver; and a higher concentration of palmitate (6%) in brain ($P<0.05$).

There was a two- to fourfold greater incorporation of ^{13}C from ALA into stearate and oleate in adipose tissue of rats on the ketogenic diet than in controls, but a 72% lower direct incorporation of ^{13}C-ALA ($P < 0.05$; Table 2). Despite the increase in liver concentrations of saturated and monounsaturated fatty acid species due to the high fat content of the ketogenic diet, recycling of ^{13}C-ALA into saturated and monounsaturated fatty acids in liver of rats that received the ketogenic diet did not differ significantly from controls ($P > 0.05$; Table 2). Moreover, the amount of ^{13}C-ALA itself incorporated into liver did not differ significantly between the two groups. In brain, carbon recycling from ^{13}C-ALA into palmitate was twofold higher in rats on the ketogenic diet ($P < 0.05$), even though brain content of palmitate was significantly raised by the high-fat ketogenic diet (Table 2).

DISCUSSION

This study shows that carbon recycling from ALA into saturated and monounsaturated fatty acids is not decreased even when intake and tissue levels of fatty acids produced during carbon recycling of ALA are raised by the high-fat ketogenic diet. Our results confirm that ALA recycling into products of *de novo* fatty acid synthesis occurs regardless of the saturated and monounsaturated fatty acid content of the diet or tissues.

ALA recycling into some, but not all, products of *de novo* fatty acid synthesis in adipose tissue and brain was more than twofold higher in rats on the ketogenic diet, despite the significant increase in the composition of stearate and oleate in adipose tissue and palmitate concentration in brain (Tables 1 and 2). The underlying pathway accounting for greater recycling of ALA, despite higher concentrations of fatty acid end products of ALA recycling, is not clear. However, greater incorporation

TABLE 2
^{13}C-ALA Enrichment in Saturated and Monounsaturated Fatty Acids of Adipose, Liver and Brain, 24 Hours after Dosing with 5 mg of ^{13}C-ALA

	Tissue:	Adipose		Liver		Brain	
	Treatment:	Control	Ketogenic Diet	Control	Ketogenic Diet	Control	Ketogenic Diet
16:0	ng 13C/g tissue	370 ± 161	155 ± 75	376 ± 142	567 ± 489	367 ± 216	780 ± 63*
18:0	ng 13C/g tissue	92 ± 32	190 ± 71*	127 ± 58	158 ± 94	250 ± 150	310 ± 13
16:1n-7	ng 13C/g tissue	68 ± 52	60 ± 70	34 ± 21	29 ± 10	NA	NA
18:1n-9	ng 13C/g tissue	452 ± 273	1964 ± 128*	116 ± 71	257 ± 165	545 ± 258	311 ± 169
18:3n-3	ng 13C/g tissue	1947 ± 591	535 ± 484*	4100 ± 1366	4891 ± 914	ND	ND

Data are the mean ± SD of n = 6/group.
16:0, palmitate, 18:0, stearate; 16:1n-7, palmitoleate; 18:1n-9, oleate; 18:3n-3, α-linolenate; NA, not available; ND, not detected.
*$P < 0.05$.

of ^{13}C-ALA into stearate and oleate in the adipose of rats on the ketogenic diet does not reflect recycling in adipose tissue, because adipose tissue has minimal capacity to oxidize fatty acids (13). It is possible, however, that ^{13}C reflects recycling in liver and subsequent uptake by adipose tissue, because the concentrations of stearate and oleate were significantly higher in the liver of rats that received the ketogenic diet, relative to control rats (Table 1).

The different fatty acid content and composition of the control and experimental diets were unlikely to affect ALA partitioning toward elongation/desaturation products of ALA, because we previously showed that rats on a ketogenic diet appear to preferentially mobilize PUFA from adipose tissue to liver and brain, yet, tracer incorporation into DHA in all these tissues remained the same (10). Our findings are consistent with other studies showing that elongation/desaturation of ALA into DHA occurs regardless of dietary or tissue concentrations of n-3 or n-6 PUFA (5,8,14,15).

We conclude that carbon recycling from ALA into *de novo* products of fatty acid synthesis persists despite higher dietary intake and tissue concentrations of saturated and monounsaturated fatty acids and low to negligible incorporation of ALA into adipose tissue and brain. It now seems clear that, under experimental conditions ranging from extreme dietary and tissue depletion of n-3 fatty acids to raised intake and tissue content of saturates and monounsaturates, carbon recycling of ALA is consistently observed (7). The biological significance underlying this quantitatively important pathway remains to be established.

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Effect of Conjugated Linoleic Acid Type, Treatment Period, and Dosage on Differentiation of 3T3 Cells

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ABSTRACT: This study was conducted to determine effect of CLA and linoleic acid (LA) on cell differentiation, cellular glycerol-3-phosphate dehydrogenase (GPDH) activity, and FA accumulation in differentiating 3T3-L1 cells (3 isomers × 3 treatment periods × 4 doses). The cells were cultured in 24-well plates for proliferation until confluence. Then they were treated with media containing 0, 10, 35, or 70 mg/L (0, 35, 125, or 250 mmol/L, respectively) of LA, *cis*9,*trans*11- or *trans*10,*cis*12-CLA during early (day 0–2), intermediate and late (day 3–8), or overall (day 0–8) differentiation periods. Dexamethasone, methyl-isobutylxanthine, and insulin were supplemented to the media only for the early period to induce the differentiation. On day 8 of postconfluence the cells were harvested for Oil Red O staining, analysis of GPDH activity, and determination of the FA concentration. Cellular LA or CLA was found to accumulate in a dose-response manner, mainly during the intermediate/late period. Treatment with *trans*10,*cis*12-CLA lowered ($P < 0.05$) GPDH activity and the concentration of FA including palmitic acid (16:0) and palmitoleic acid (16:1), especially during the intermediate/late and overall periods, or whenever a high dose of 70 mg/L was applied. This also resulted in a higher ($P < 0.05$) ratio of saturated FA to monounsaturated FA. Treatment with LA or *cis*9,*trans*11-CLA lowered cellular FA only when they applied during the early period at a dose of 70 mg/L. The results demonstrated that the inhibitory effects of CLA on differentiation, GPDH activity, and FA accumulation of 3T3-L1 cells are dependent on the isomer type, treatment period, and dose.

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CLA are positional and geometric isomers of linoleic acid (LA) produced during biohydrogenation of FA by rumen bacteria such as *Butyrivibrio fibrisolvens* (1) or in body tissues from *trans*-11 octadecenoic acid by Δ^9-desaturase (2). In animal models, dietary CLA has induced positive effects that could be applied to enhance human health (3). Studies found that a mixture of synthetic CLA containing isomers of *cis*9,*trans*11 (c9,t11) and *trans*10,*cis*12 (t10,c12) decreased body fat mass in rats (4), a result that possibly reflected increasing energy expenditure (5) or decreasing adipocyte number (6). The effects of c9,t11- and t10,c12-CLA may be different on lipogenesis and adipogenesis of adipose tissues. It was reported that t10,c12-CLA had a better effect on decreasing body fat in mice than c9,t11-CLA (7).

Early *in vitro* studies found that a mixture of CLA in culture medium inhibited preadipocyte differentiation (8,9) but stimulated lipogenesis in 3T3-L1 preadipocytes (9). In studies using pure CLA, t10,c12-CLA attenuated lipogenesis in primary human adipocytes (10) and increased FA oxidation in 3T3-L1 cells (11). Brown *et al*. (12,13) reported that t10,c12-CLA decreased cellular TAG accumulation in differentiating human preadipocytes, and it functioned similarly in 3T3-L1 cells (14–16). However, McNeel *et al*. (17) found that cellular TAG concentration, glycerol-3-phosphate dehydrogenase (GPDH) activity, and peroxisome proliferator-activated receptor γ (PPARγ) were increased in either c9,t11- or t10,c12-CLA-treated human preadipocytes.

3T3-L1 cell lines have been used as models for study on the differentiation from preadipocytes to adipocytes (18). Dexamethasone, methyl-isobutylxanthine, and insulin (DMI) typically were used to induce the differentiation of the cells (19). Within 2 d of treatment with media containing DMI, the cells completed a postconfluent mitosis and formed a growth arrest. Treatment with DMI allowed the expression of transcription factors including PPARγ, which could reach a high level on day 4 of postconfluence, at which time the differentiation generally was completed (19). Therefore, the differentiating periods could be divided into early (day 0–2), intermediate (day 2–3), and late (from day 4) periods according to the appearances of differentiation markers. It is necessary to clarify the effect of CLA or LA on the cells in various differentiation periods and the doses applied in previous studies (13,14,16,17).

The present study was conducted to investigate the effects of LA, c9,t11- or t10,c12-CLA, of their treatment periods, and of their doses on the differentiation process in DMI-induced differentiating 3T3-L1 preadipocytes. As main indicators of differentiation and lipid accumulation, the Oil Red O-stained cell profile, cellular GPDH (EC 1.1.1.8) activity, and the concentration of the main cellular FA were compared among various treatments.

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Abbreviations: DHAP, dihydroxyacetone phosphate; DMI, dexamethasone, methyl-isobutylxanthine, and insulin; GPDH, glycerol-3-phosphate dehydrogenase; LA, linoleic acid; MUFA, monounsaturated fatty acid(s); PPARγ, peroxisome proliferator-activated receptor γ; SCD, stearoyl-CoA desaturase; SFA, saturated fatty acids.

MATERIALS AND METHODS

Experimental design and cell culture. This is a 3 FA × 3 treatment periods × 4 doses cell culture experiment. The 3T3-L1 cell line was obtained from the American Type Culture Collection (Manassas, VA). Passages of the cells used in present study were seven or eight. The cells were seeded to 24-well plates at a density of 1.5×10^4 cells per mL of DMEM (Sigma Chemical, St. Louis, MO) containing 10% FBS (Sigma Chemical) in a humidified 5% CO_2 atmosphere at 37°C for proliferation until confluence was attained. The total 432 wells of confluent cell cultures were treated, respectively, with media containing 0, 10, 35, or 70 mg/L (0, 36, 125, 250 μmol/L, respectively) of LA, c9,t11-, or t10,c12-CLA during the early (day 0–2), intermediate/late (day 3–8), or overall (day 0–8) differentiation period. Each treatment included 12 replications. The media contained 0.1% ethanol used as carrier for FA. LA was obtained from (Sigma Chemical), and isomers of CLA were obtained from Matreya, Inc. (Pleasant Gap, PA) with a purity of more than 98% for both. The basic media for the intermediate/late period (day 3–8) was DMEM supplemented with 10% FBS. All the media including the control and the FA treatments during the early period (day 0–2) were supplemented with 0.1 μmol/L dexamethasone (Sigma Chemical), 1 mmol/L methyl-isobutylxanthine (Sigma Chemical), and 0.1 μmol/L insulin (bovine insulin; Sigma Chemical) that acted as the main factors (DMI) to induce initial differentiation.

Harvesting and counting cells. At the end of culture experiment (day 8), the cells in 5 wells per treatment (total 140 wells) were harvested for analysis of GPDH activity whereas the contents of the other 140 wells were harvested for FA analysis. The remaining 56 wells were stained with Oil Red O and Mayer's hematoxylin (Sigma Chemical) before being photographed. For counting and for analysis of FA, cells were harvested by washing them with warm PBS (pH 7.08) and then dissociating by incubation with PBS containing 0.25% trypsin and 100 mg/L EDTA. The cells were counted on a hemocytometer. For analysis of GPDH activity, the cells were washed with cold PBS (calcium- and magnesium-free). Then 0.5 mL Tris-EDTA (pH 7.5) was added to each well. The cells were collected mechanically and fractured by ultrasonication. After centrifugation at 12,800 × *g* for 5 min at <5°C, the supernatant was taken for analysis of GPDH activity. All the harvested cells and extracts were stored at –80°C until further analysis.

Staining and photographing cells. The cells, in duplicate per treatment (total 56 wells), were stained with Oil Red O and Mayer's hematoxylin. Briefly, 1 mL of cold, buffered formalin was added to the cell culture and kept at room temperature for 30 min. Then it was replaced with fresh 2 mL of cold, buffered formalin for 1 h at room temperature to achieve fixation. After washing with deionized water, 1 mL of 0.5% Oil Red O (Sigma Chemical) in isopropanol/deionized water (3:2) was added to the fixed cells for 1 h to stain the oil bodies in the cells. Then the cells were washed with deionized water, then counterstained with Mayer's hematoxylin (1 g/L; Sigma Chemical) for 3 min, and washed with deionized water again. The cell cultures were photographed by using a microscope digital camera.

GPDH (EC 1.1.1.8) activity and protein analysis. The GPDH activity assay was carried out following a previous method, with modifications (20). Cellular extract (100 μL) was added to mixture of the following: 50 μL of TEA solution (pH 7.5: composed of 0.5 M triethanolamine, 10 mM EDTA, and 10 mM 2-mercaptoethanol); 100 μL of 5 mmol/L dihydroxyacetone phosphate (DHAP); and 200 μL 0.5 mM NADH. The disappearance of NADH at 25°C was measured by a spectrophotometric method at 340 nm with a microplate spectrophotometer (Benchmark Plus; Bio-Rad, Hercules, CA). The activity of GPDH was expressed as units per min = [NADH (100 nmol) × change of OD_{340}]/[1.25 × time (min) × protein (mg)]. Protein content was measured with the modified method of Lowry *et al.* (21) with a modified Lowry protein assay kit (Pierce, Rockford, IL).

Extraction of cellular lipids. The cellular lipids were extracted by using a procedure reported by Jiang *et al.* (22) with modifications. The harvested cells were transferred into a test tube to which 7 mL of isopropanol was added and then homogenized for 3 min. Next, 5 mL of hexane was added and mixed, followed by 4 mL of water, and the tube contents were homogenized again. The mixture was then centrifuged at 2,000 × *g* at 5°C for 5 min. The upper layer was transferred to a new tube. The extraction was repeated twice with hexane. Then 7 mL of 0.47 M aqueous Na_2SO_4 was added to the extracted liquid and mixed. The solution was centrifuged again at 2,000 × *g* at 5°C for 5 min. The hexane layer was collected into a tube and evaporated under nitrogen in a 38°C water bath. The residue was flushed with nitrogen and stored at –80°C for further analysis.

Methylation and determination of FA. A combined base/acid methylation method (23) with modifications was used. Heneicosanoic acid (21:0) methyl ester was used as an internal standard by adding it to the tubes containing extracted cellular lipids (75 μL, 5 mg/mL Nu-Chek-Prep, Inc., Elysian, MN). Then 2 mL of sodium methoxide (0.5 mmol/L in methanol) was added to each tube, each tube was mixed completely and flushed with nitrogen, then placed in a 50°C water bath for 10 min. After that, 1 mL of boron trifluoride (14% in methanol) was added to each tube, and the tube reheated in a 50°C water bath for another 10 min. Then 5 mL water and 1 mL hexane were added and mixed completely. After standing for 10 min. the upper layer (hexane) was taken into a GC vial flushed with nitrogen and stored at –30°C until FA profiles were determined by GC. The FA profiles were analyzed on a Shimadzu gas chromatograph (GC-14B; Shimadzu Co., Kyoto, Japan) with an Rtx®-2330 capillary column (Bellefonte, PA; 30 m × 0.32 mm i.d. × 0.2 μm film thickness). The initial GC temperature was 100°C, then increased to 175°C at a rate of 25°C/min. After holding at 175°C for 5 min, the temperature was increased to 225°C at a rate of 5°C per min and held for 10 min. Helium was used as the carrier gas. The FA were identified by comparison with retention times of known FAME standards. The concentration of identified FA was then calculated based on the ratio of the FA to the internal standard.

Statistical analysis. ANOVA was carried out to determine the effect of the main treatments—LA and isomers of CLA, treatment periods, and doses—and their interactions on the cellular GPDH activity, FA concentration, and the ratio of satu-

rated FA to monounsaturated FA (24). ANOVA was also applied for analysis of the effect among all the combinations of the treatment periods and doses within the same LA or CLA, or among the LA and CLA. Tukey multiple tests were used to compare the means of the treatments. Analysis was made to determine the correlation between cellular GPDH activity and FA concentration, and between cellular GPDH activity and the ratio of saturated FA/monounsaturated FA (24). The significance level was set as $P < 0.05$.

RESULTS

Profile of Oil Red O-staining cells. The cells in the control group were generally differentiated to adipocytes, as indicated by a large proportion of Oil Red O-stained cells shown in the photographs (Fig. 1). Treatment with media containing DMI for 2 d (day 0–2), followed by a further culture in FBS-supplemented DMEM for lipid accumulation (day 3–8), could effectively induce differentiation and lipid accumulation in the confluent 3T3-L1 cells. Supplementation with t10,c12-CLA (10–70 mg/L) during the overall differentiation period (day 0–8) resulted in a much lower proportion of Oil Red O-stained cells than all the others did. Similarly, the treatment with t10,c12-CLA (10–70 mg/L) during the intermediate/late differentiation period (day 3–8) also led to a much lower proportion of Oil Red O-staining cells than all the others did. The treatment with a high dose (70 mg/L) of LA or CLA only during the early period also apparently produced a relative low proportion of Oil Red O-stained cells. However, the treatment with a high concentration of c9,t11-CLA (35 or 70 mg/L) during the overall period (day 0–8) led to a higher proportion of Oil Red O-staining cells containing large lipid drops compared with the others (Fig. 1).

Cellular GPDH activity. The cellular GPDH activity was significantly lowered in cells treated with 70 mg/L of t10,c12-CLA during the early and intermediate/late periods, and in those treated with doses of 35 and 70 mg/L during the overall period (Fig. 2). The cellular GPDH activities in the treatments under above conditions were only about half that of the control. However, the treatments with LA (35 and 70 mg/L) and c9,t11-CLA (10–70 mg/L) during the overall period led to a significantly higher cellular GPDH activity than that in the control. There was no significant difference in the cellular GPDH activity between the control and the treatments with LA and

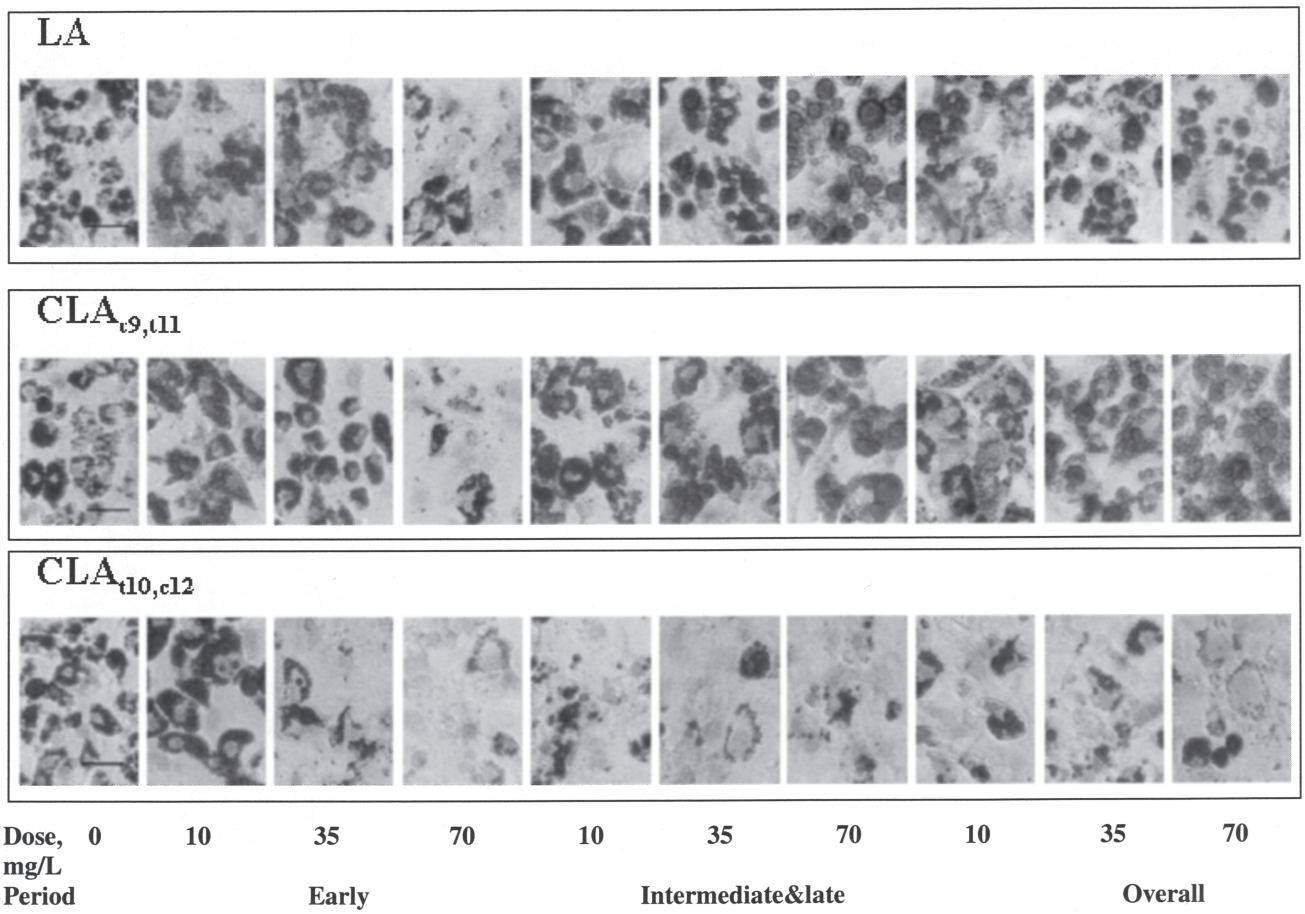

FIG. 1. Photographs of 3T3-L1 adipocytes on day 8 of postconfluence after treatment with or without various doses of CLA or linoleic acid (LA) during differentiation periods (Early: days 0–2; intermediate&late, days 3–8; overall, days 0–8). Cells were stained with Oil Red O and Mayer's hematoxylin. Bar = 50 μm.

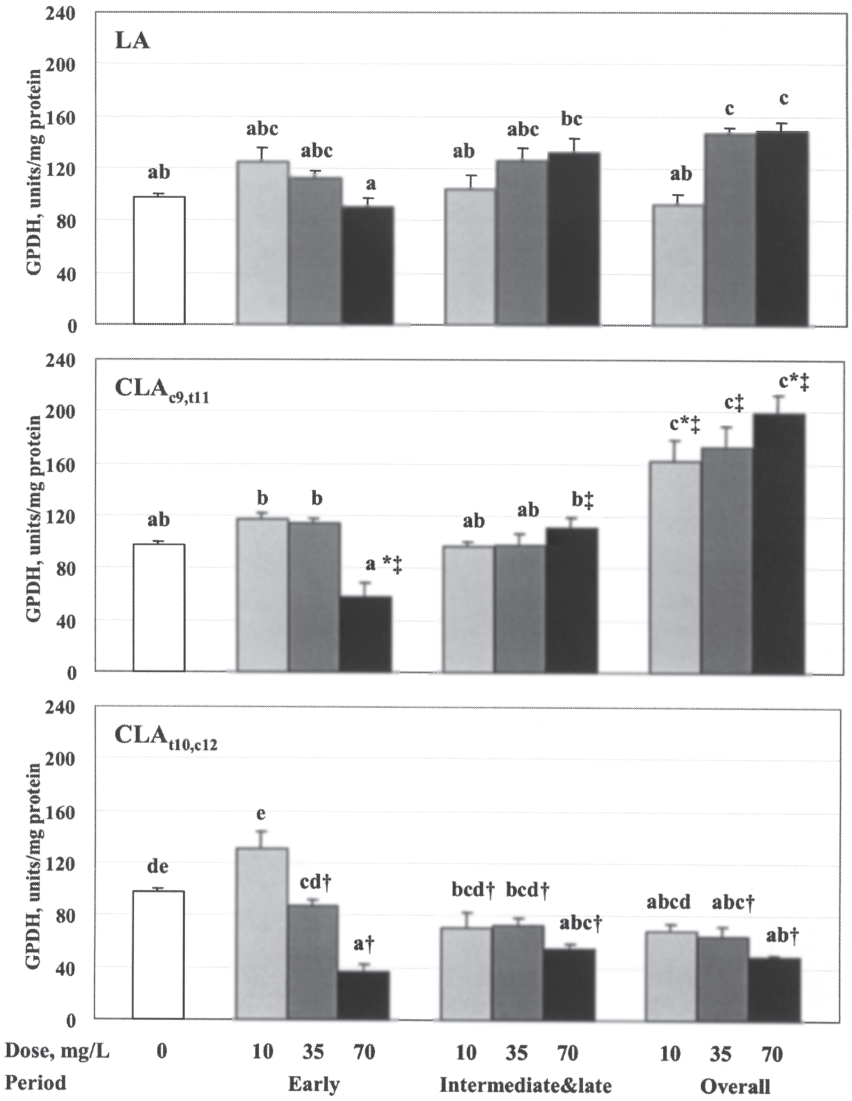

FIG. 2. Activity of glycerol-3-phosphate dehydrogenase (GPDH: EC1.1.1.8) in the 3T3-L1 adipocytes after treatment with or without various CLA or LA dosages during differentiation periods. In the groups treated with the same CLA or LA dosage, means (±SE, $n = 5$) without the same letter (a–e) differ significantly ($P < 0.05$). *, †, and ‡ indicate a significant difference when comparison is made of c9,t11-CLA vs. LA; t10,c12-CLA vs. LA; and c9,t11-CLA vs. t10,c12-CLA, respectively ($P < 0.05$). For abbreviation and terminology regarding periods see Figure 1.

c9,t11-CLA during the early and intermediate/late periods, although the activity in cells treated with 70 mg/L LA or c9,t11-CLA during the early period were relatively lower than that of the control.

A significant difference was also found in the GPDH activity between CLA and LA treatments (Fig. 2). Comparison of these treatments was based on the same treatment period with same dose. GPDH activities in the treatments of t10,c12-CLA were significantly lower than those of LA when doses of 35 and 70 mg/L were applied. However, treatment with c9,t11-CLA led to a higher ($P < 0.05$) GPDH activity compared with that of LA, especially when applied during the overall period.

Viability of the cells. The average density of the cells harvested on day 8 of postconfluence was between 9.5×10^4 and 12×10^4 cells/mL, which was six- to eightfold higher than that of cells on day 0 at the beginning (1.5×10^4 cells/mL). The treatment with CLA and LA during the various periods of differentiation did not significantly affect the final cell number, except relatively higher values were found for LA and c9,t11-CLA when they were applied during the intermediate/late period with a dose of 70 mg/L (Fig. 3).

Accumulation of LA or CLA. The accumulation of LA or CLA in cells treated with the corresponding FA was dose-dependent (Fig. 4). The accumulations of LA or CLA in the cells treated during the intermediate/late period were close to that in the cells treated during the overall period (day 0–8). However,

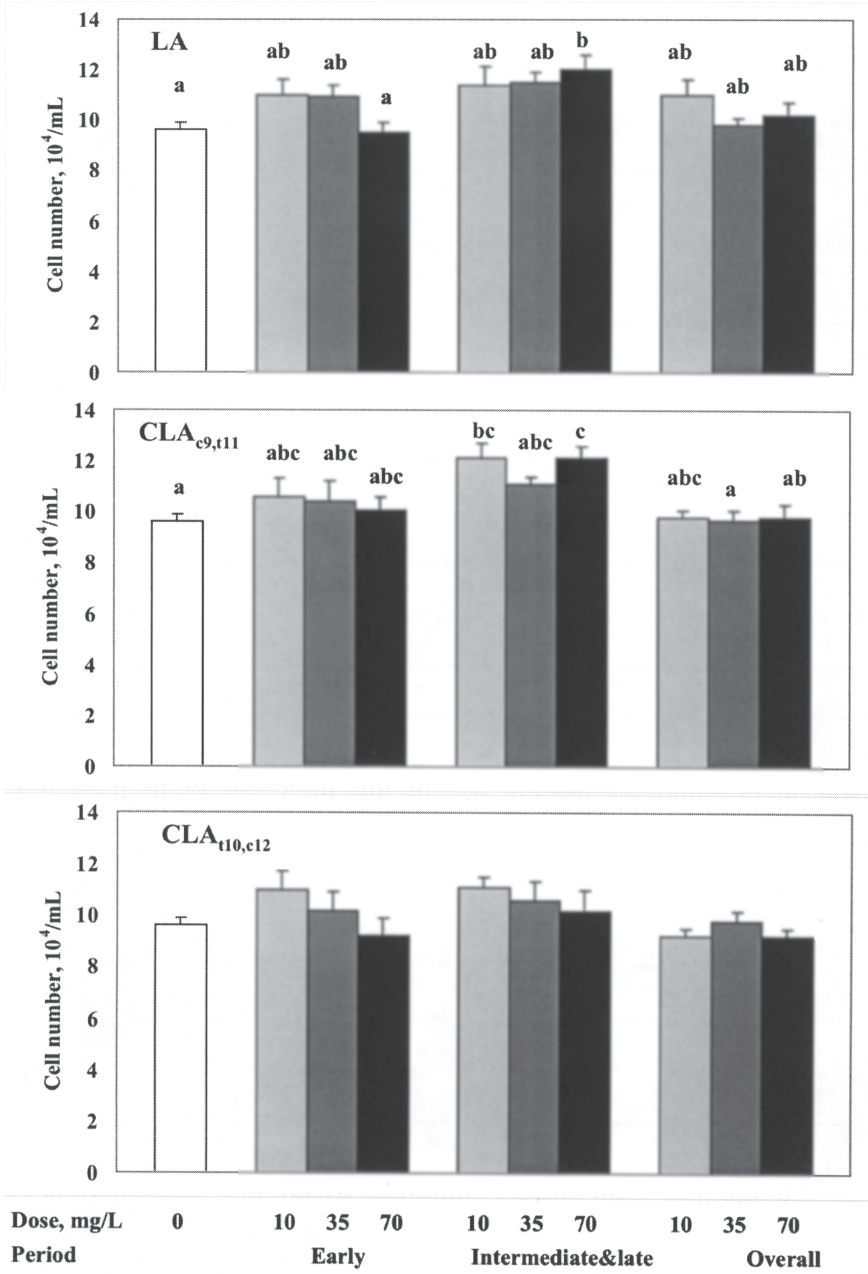

FIG. 3. Viability of cells after treatment with or without various CLA or LA dosages during differentiation periods. In groups treated with the same CLA or LA dosage, means (±SE, $n = 8$) without the same letter (a–c) differ significantly ($P < 0.05$). For abbreviation and terminology regarding periods see Figure 1.

the cells treated during the early period were very low in cellular concentrations of LA or CLA. The cellular concentrations of t10,c12-CLA were only about one-third of the LA or c9,t11-CLA accumulated in the cells treated with corresponding FA isomers, although the same dose was applied.

Concentration of main cellular FA. Comparisons of the sum of the main FA and of palmitic acid (16:0) and palmitoleic acid (16:1) are shown in Figures 5, 6, and 7, respectively.

The treatment with t10,c12-CLA during the overall and intermediate/late periods significantly decreased the total amount of the FA (Fig. 5), 16:0 (Fig. 6), and 16:1 (Fig. 7). The cellular concentration of 16:1 in the cells under these conditions was only 5–20% of that in the control group. The treatment with t10,c12-CLA during the overall (70 mg/L) and intermediate/late (35 and 70 mg/L) periods significantly decreased the cellular concentration of 16:0 FA (Fig. 6). However, treatment with 10 and 35 mg/L doses of t10,c12-CLA during the early period (day 0–2) had little effect on the synthesis of the main FA but with a 70 mg/L dose the FA and 16:0 and 16:1 FA were greatly lowered (Figs. 5–7).

The treatment with c9,t11-CLA during intermediate/late (35 and 70 mg/L) and the overall periods significantly increased

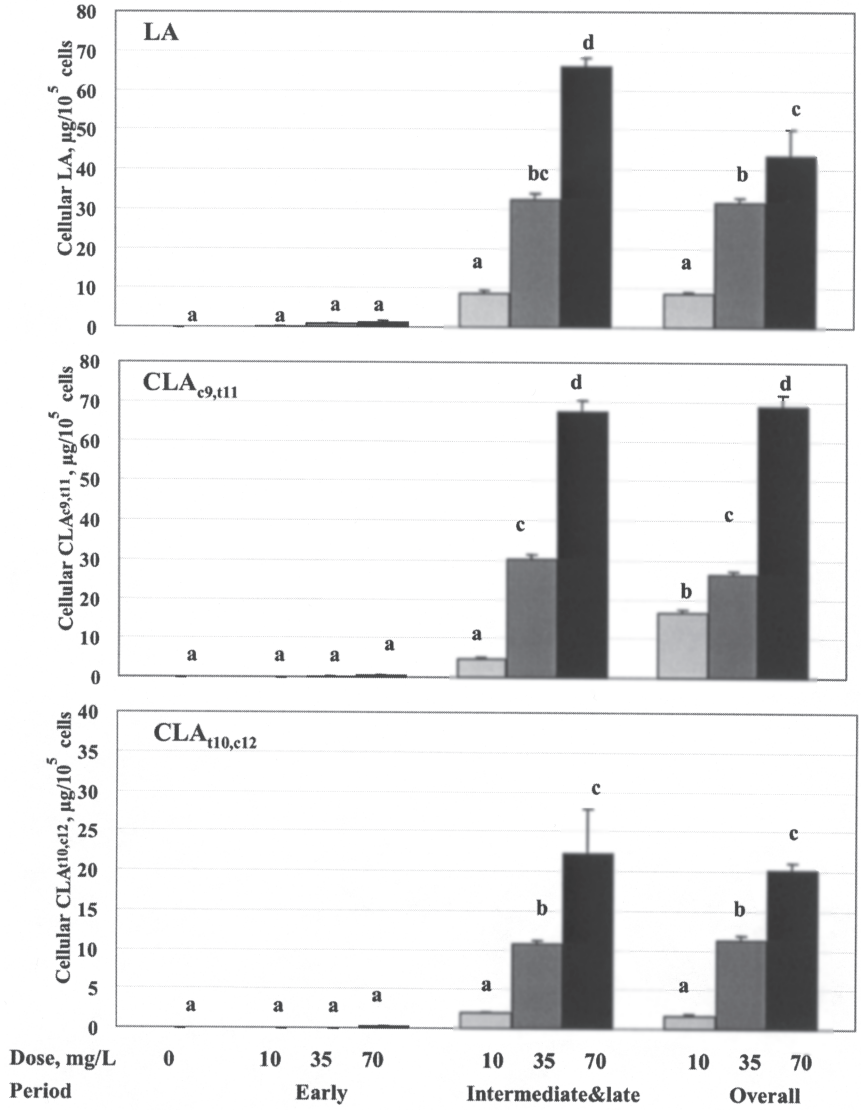

FIG. 4. Concentration of CLA or LA in the 3T3-L1 adipocytes after treatment with or without various CLA or LA dosages during differentiation periods. In groups treated with the same CLA or LA dosage, means (±SE, $n = 5$) without the same letter (a–d) differ significantly ($P < 0.05$). For abbreviation and terminology regarding periods see Figure 1.

the cellular amount of both the total and individual FA, especially that of 16:0 FA (Figs. 5–7). The concentration of 16:0 in the treatment with c9,t11-CLA was two- to threefold higher than that in the control when c9,t11-CLA was applied at doses of 35 and 70 mg/L during the overall and intermediate/late periods. The treatment with c9,t11-CLA during the early period did not significantly change the concentration of the main FA in the cells compared with that of the control, although a high dose of 70 mg/L had a tendency ($P < 0.1$) to decrease the cellular FA accumulation.

The treatment with LA during the overall (70 mg/L) and intermediate/late periods also significantly increased the amounts of total FA and 16:0 (Figs. 5 and 6). However, the treatment with a 70 mg/L dose during the early period significantly lowered the main FA accumulation in the cells, along with the decrease in the cellular 16:1 FA.

When comparison was made based on the same treatment period and dose, the treatments with t10,c12-CLA produced significantly lower cellular concentrations of the total or individual FA (Figs. 5–7) than with LA-treated cells during the overall and intermediate/late periods. By contrast, the treatments with c9,t11-CLA generally resulted in a higher cellular FA concentration than that of LA-treated cells during the overall period.

*Ratios of 16:0/16:1 and 18:0/18:1 FA***.** Treatment with LA and CLA doses of 10 and 35 mg/L during the early period had little effect on the ratios of 16:0/16:1 and 18:0/18:1, which ranged from1.4 to 1.8 (Fig. 8) and from 0.21 to 0.26 (Fig. 9),

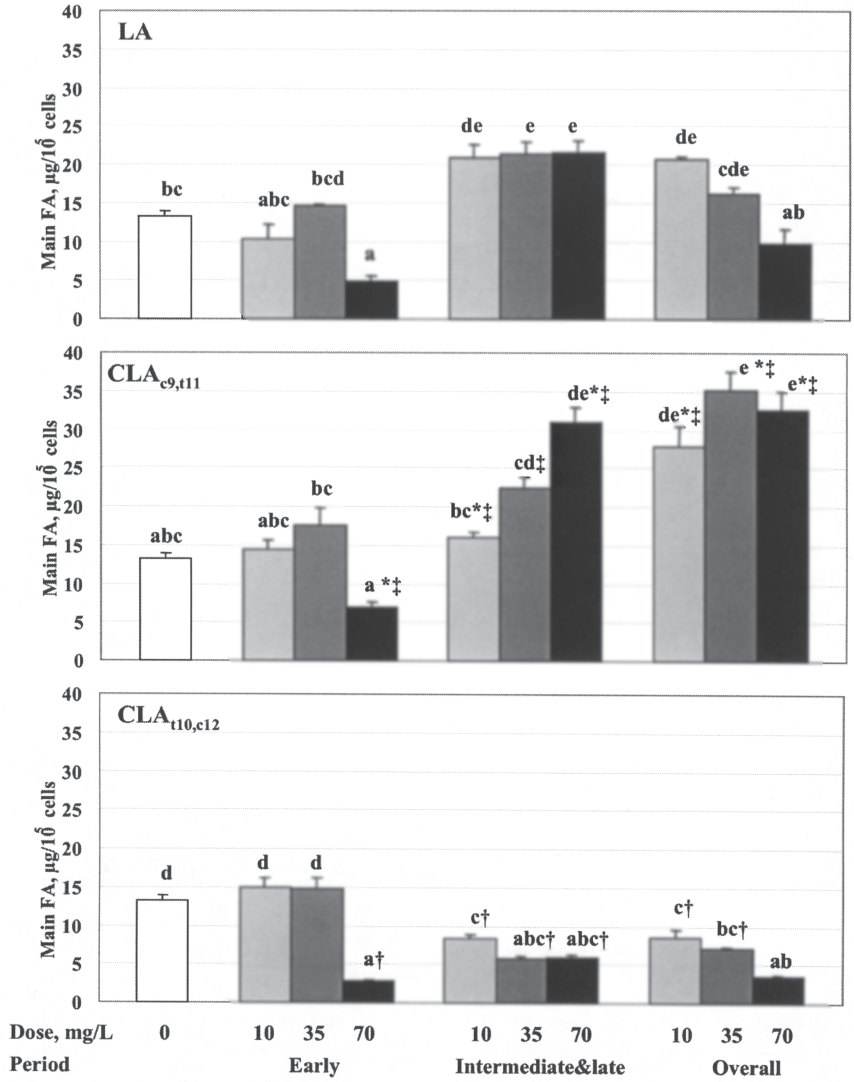

FIG. 5. Main FA in the 3T3-L1 adipocytes after treatment with or without various CLA or LA during differentiation periods. The "main FA" represent the sum of 16:0, 16:1, 18:0, and 18:1 detected in cellular lipids. In groups treated with the same CLA or LA dosage, means (±SE, n = 5) without the same letter (a–e) differ significantly ($P < 0.05$). *, †, and ‡ indicate a significant difference when comparison is made of c9,t11-CLA vs. LA; t10,c12-CLA vs. LA; and c9,t11-CLA vs. t10,c12-CLA, respectively ($P < 0.05$). For abbreviation and terminology regarding periods see Figure 1.

respectively. However, treatment with t10,*c12-CLA* during the overall and intermediate/late periods increased the ratios of 16:0/16:1 and 18:0/18:1 to 7.8-22 and 0.55-0.77, respectively. The treatment with t10,c12-CLA using a high dose of 70 mg/L during the early period also increased the ratios of 16:0/16:1 and 18:0/18:1 to 7.7 and 0.60 respectively. The treatment with a c9,*t11-CLA* dose of 10 or 35 mg/L during the overall and intermediate/late periods only moderately increased the ratios, wh*ereas* treatment with a dose of 70 mg/L significantly increased the ratios. Similarly, the treatment of LA during the overall and intermediate/late periods only led to a moderate increase in those ratios when a normal dose of 10 mg/L was applied. However, the ratios were significantly increased when a high dose of 70 mg/L was applied.

Correlation between cellular GPDH activity and accumulation of FA. Significant positive correlations were observed between the GPDH activity and the mainly synthesized FA concentration. Most of the coefficients were larger than 0.7 (Table 1). A significant negative correlation was observed between the GPDH activity and ratio of 16:0/16:1 and of 18:0/18:1, with correlation coefficients of –0.55 and –0.44, respectively.

DISCUSSION

The present study showed that t10,c12-CLA depressed differentiation and cellular lipid accumulation in differentiating 3T3-L1 cells, whereas c9,t11-CLA and LA enhanced them under certain conditions. The results are consistent with previous re-

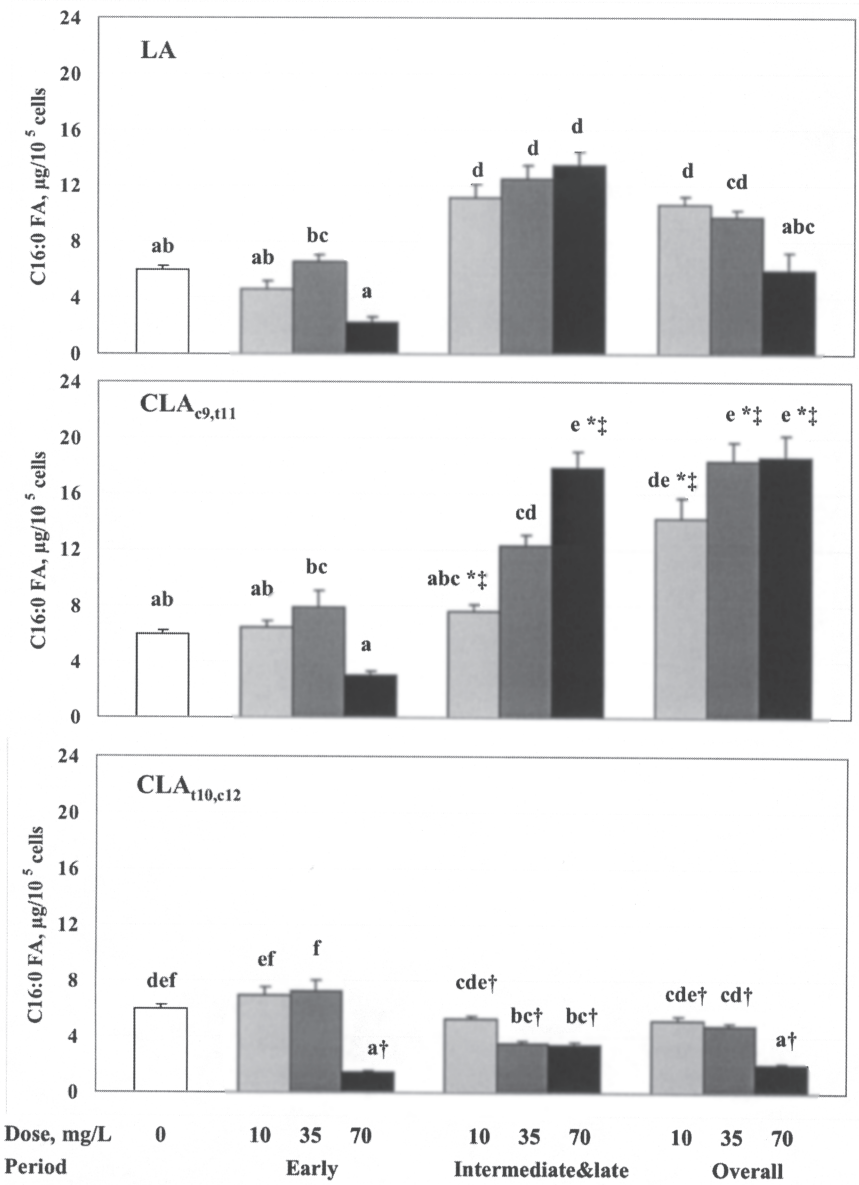

FIG. 6. Palmitic acid (16:0) in the 3T3-L1 adipocytes after treatment with or without various CLA or LA dosages during differentiation periods. In groups treated with the same CLA or LA dosage, means (±SE, $n = 5$) without the same letter (a–e) differ significantly ($P < 0.05$). *, †, and ‡ indicate a significant difference when comparison is made of c9,t11-CLA vs. LA; t10,c12-CLA vs. LA; and c9,t11-CLA vs. t10,c12-CLA, respectively ($P < 0.05$). For abbreviation and terminology regarding periods see Figure 1.

ports of *in vivo* studies showing that dietary CLA, especially t10,c12-CLA, lowered the body fat mass in animals and humans (7,25,26). The contrasting effects of these two CLA isomers were also seen in FA profiles and concentration of liver lipids from rats and mice (27,28). The results are in agreement with those studies using primary cultures of stromal vascular cells from human adipose tissue (10,13), which indicated that t10,c12- but not c9,t11-CLA decreased both the total TAG concentration and lipogenesis in the cells. Similarly, Kang *et al.* (14) reported that t10,c12-CLA inhibited differentiation of 3T3-L1 cells and resulted in lower TAG in the cells compared with the control. However, an *in vitro* study using human preadipocytes showed that c9,t11- and t10,c12-CLA increased differentiation and TAG concentration of the cells (17). The different effects found in *in vivo* studies may be due to the species and the degree of obesity in the animals whereas in *in vitro* studies, the differences may be related to treatment timing, length, dose, and the variation in cellular amount of incorporated CLA.

GPDH is the most important enzyme to catalyze the conversion of DHAP to glycerol 3-phosphate, which is a predominant substrate for TAG biosynthesis in adipocytes (20). GPDH was

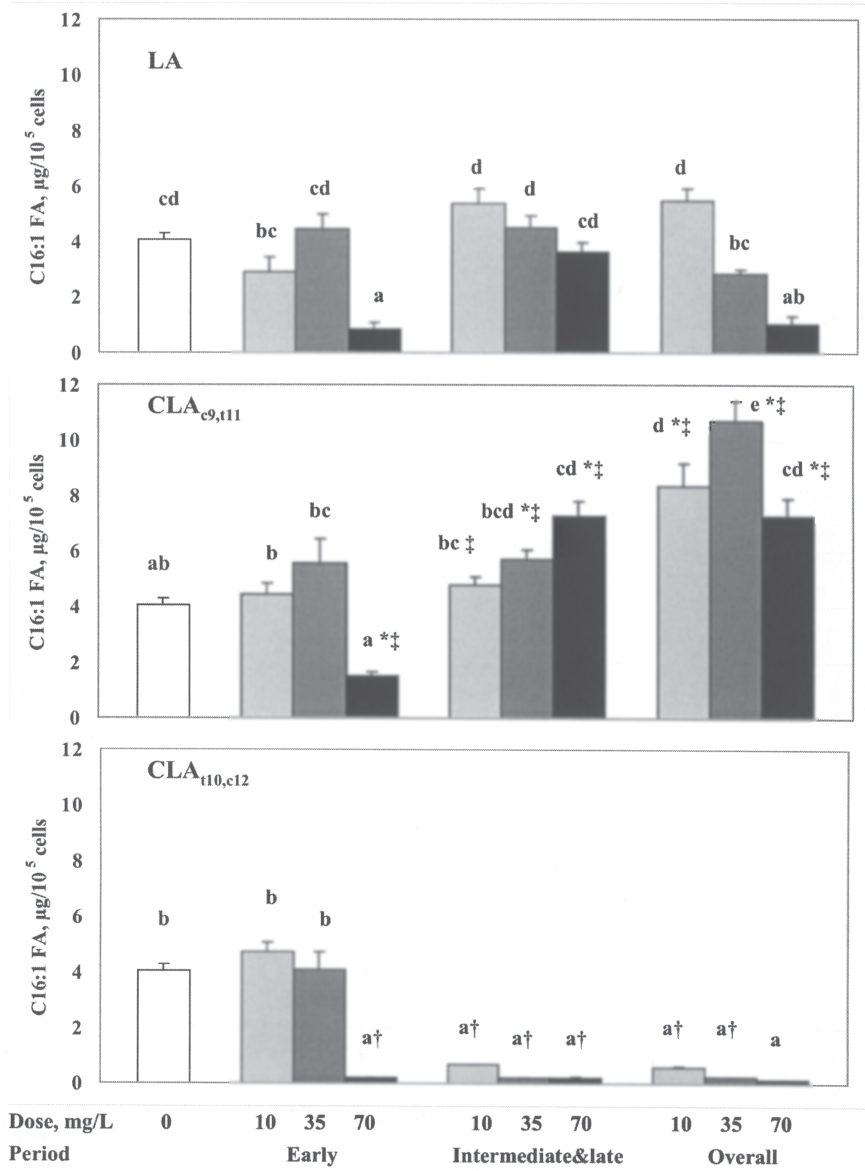

FIG. 7. Palmitoleic acid (16:1) in the 3T3-L1 adipocytes after treatment with or without various CLA or LA dosages during differentiation periods. In groups treated with the same CLA or LA dosage, means (±SE, $n = 5$) without the same letter (a–e) differ significantly ($P < 0.05$). *, †, and ‡ indicate a significant difference when comparison is made of c9,t11-CLA vs. LA; t10,c12-CLA vs. LA; and c9,t11-CLA vs. t10,c12-CLA, respectively ($P < 0.05$). For abbreviation and terminology regarding periods see Figure 1.

measured because it is a very important biochemical marker for the differentiation of preadipocyte to adipocyte and because its activity and gene expression increased greatly during the process (20,29). This study found that GPDH activity had a very significant ($P < 0.001$) positive correlation with the main synthesized FA in the cells. The treatment with a high dose of 35–70 mg/L of t10,c12-CLA during any of the differentiation periods led toa decrease in GPDH activity, whereas treatment with c9,t11-CLA and LA led to an increase. The effects are similar to those found in human adipocytes, in which chronic treatment with t10,c12-CLA resulted in a time-dependent decrease in mRNA expression of GPDH gene but not with c9,t11-CLA treatment (13).

In the present study, the FA concentration was measured as the main indicator of cellular lipid accumulation including those FA synthesized and incorporated from media. This method has been used in previous studies (9,30). The amount of CLA or LA incorporated into the cells was found to vary with timing and length of administration of the treatments and dosage. The concentration of CLA or LA incorporated into the cells increased as the dose applied increased. Treatment in the early period led to a very low accumulation of LA or CLA at

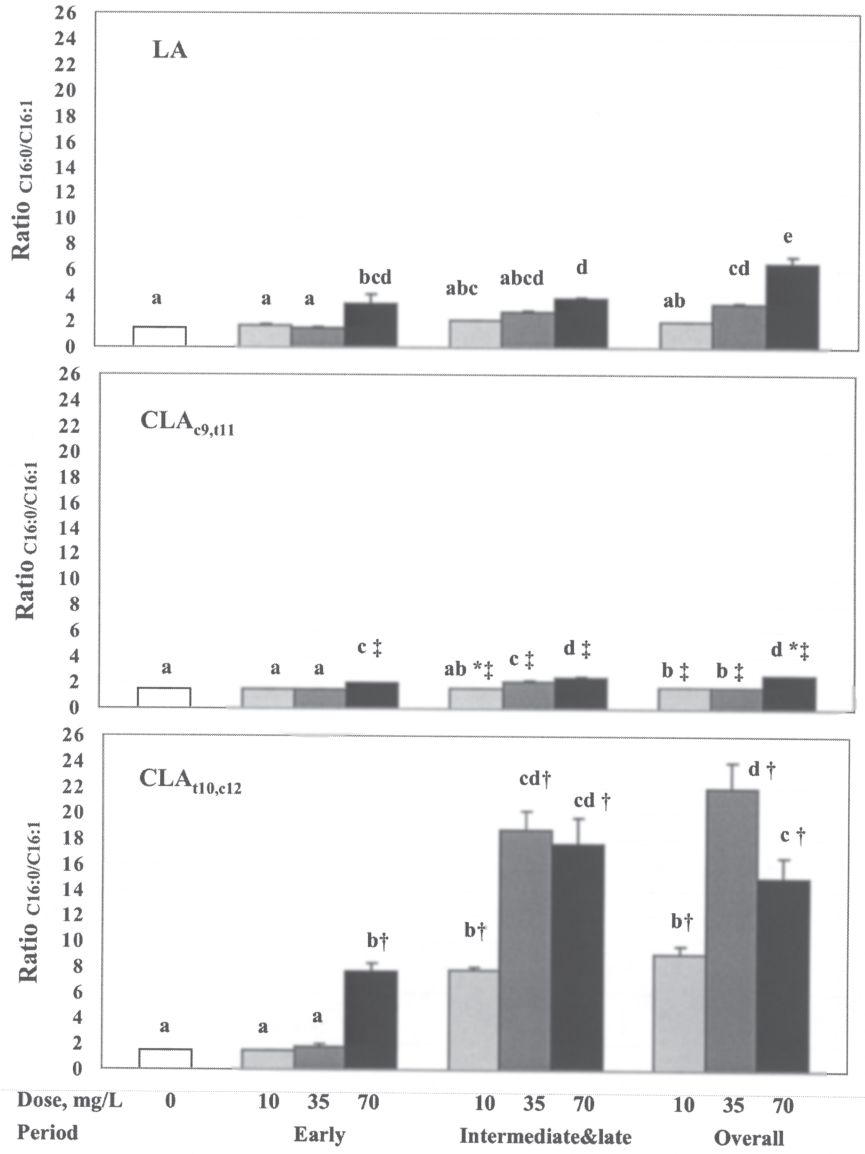

FIG. 8. Ratios of 16:0 to 16:1 FA in the 3T3-L1 adipocytes after treatment with or without various CLA or LA dosages during differentiation periods. In groups treated with the same CLA or LA dosage, means (±SE, $n = 5$) without the same letter (a–e) differ significantly ($P < 0.05$). *, †, and ‡ indicate a significant difference when comparison is made of c9,t11-CLA vs. LA; t10,c12-CLA vs. LA; and c9,t11-CLA vs. t10,c12-CLA, respectively ($P < 0.05$). For abbreviation and terminology regarding periods see Figure 1.

the end of experiment, which may be caused by the low incorporation of the FA and/or the possibility of turnover in the intermediate/late period. The amount of the main cellularly synthesized FA more reasonably reflected the *de novo* synthesis of lipids without being affected by the amount of CLA or LA incorporated directly from the media. It was also possible to calculate and compare the ratio of saturated FA/monounsaturated FA among cells treated with or without CLA through measuring the concentration of cellular individual FA. The FA ratios for 16:0/16:1 and 18:0/18:1 may relate to metabolic rate and physiological responses of the cells, because changes in these ratios affect membrane fluidity (31).

t10,c12-CLA decreased PPARγ and the expression of some adipogenic marker genes in 3T3-L1 adipocytes (14,16) and in human adipocytes (12,13), although a contradictory report indicated CLA increased the expression of these genes (17). The inhibition effect of t10,c12-CLA on the lipid accumulation of the adipocytes may be attributable to its specialized molecular structure. The small change in the structure of t10,c12-CLA, such as a-methylation, completely abolished its inhibitory effect on TAG accumulation and the expression of adipogenic marker genes (32). The decrease in FA concentration induced by t10,c12-CLA may be also due to an increase in FA oxidation in the cells (11). The present study found that the ratio of

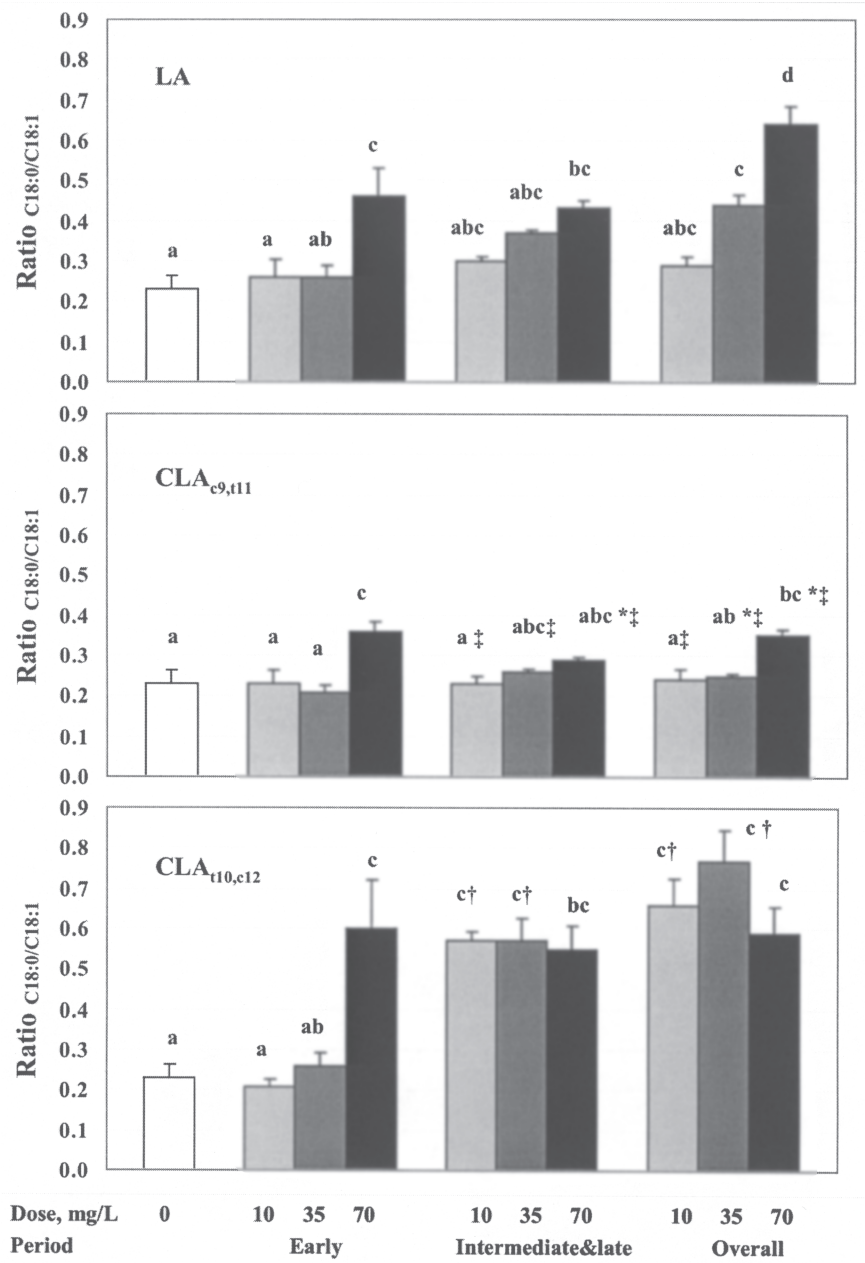

FIG. 9. Ratios of 18:0 to 18:1 FA in the 3T3-L1 adipocytes after treatment with or without various CLA or LA dosages during differentiation periods. In groups treated with the same CLA or LA dosage, means (±SE, $n = 5$) without the same letter (a–e) differ significantly ($P < 0.05$). *, †, and ‡ indicate a significant difference when comparison is made of c9,t11-CLA vs. LA; t10,c12-CLA vs. LA; and c9,t11-CLA vs. t10,c12-CLA, respectively ($P < 0.05$). For abbreviation and terminology regarding periods see Figure 1.

saturated FA to monounsaturated FA was greatly increased in cells treated with t10,c12-CLA. The results are consistent with those previous reports on t10,c12-CLA, such as its down-regulation of stearoyl-CoA desaturase (SCD) 1 gene expression and the inhibition of SCD activity in the cells (12,33).

Treatment with c9,t11-CLA under most of the experimental conditions used in the present study did not depress GPDH activity or FA accumulation in the cells. By contrast, treatment with c9,t11-CLA improved these, as did LA under certain conditions. Some of the previous studies found that c9,t11-CLA did not affect TAG accumulation and the expression in adipocytes of related genes (11,15), whereas others found that treatment with this CLA isomer increased TAG concentration, as did LA (13,14,17). The FA or their derivatives could be involved in the differentiation of preadipocytes to adipocytes as signal transduction molecules (34) and the gene expression of

TABLE 1
Correlations Between Cellular GPDH Activity and Concentration of FA or Ratio of SFA to MUFA in the 3T3-L1 Adipocytes After Treatment With or Without Various CLA or LA During Differentiation Periods[a] (*n* = 28)

Covariates	Coefficient	Significance of correlation
GPDH – 16:0 FA	0.76	<0.001
GPDH – 16:1 FA	0.73	<0.001
GPDH – 18:0 FA	0.65	<0.001
GPDH – 18:1 FA	0.78	<0.001
GPDH – 20:4 FA	0.44	0.018
GPDH – 22:6 FA	0.72	<0.001
GPDH – Ratio of 16:0/16:1 FA	–0.55	0.003
GPDH – Ratio of 18:0/18:1 FA	–0.44	0.018

[a]LA, linoleic acid; GPDH, glycerol-3-phosphate dehydrogenase; SFA, saturated FA; MUFA, monounsaturated FA.

the FA-binding protein aP-2 (35) or activate PPAR in the cells (36). They could be potential ligands for PPARÁ, the important adipogenesis marker mainly expressed during the intermediate/late differentiation period.

The timing, length, and dose of the treatment with CLA or LA affect cell differentiation. Effective doses of CLA or LA used in previous studies for cell culture were 25–100 μmol/L (13,14,16,17). The CLA or LA in the media could be incorporated into cellular TAG directly, which is confirmed in the present experimental conditions. Variation of incorporated CLA may affect the comparison result, when the cellular TAG was applied as the main lipid accumulation factor. Treatment with a relatively low concentration of CLA during the early period did not affect the differentiation and the accumulation of lipids. Similar results were found in PPARγ expression in 3T3-L1 cells treated with CLA (32). However, a high dose (70 mg/L) of CLA or LA depressed GPDH activity and FA accumulation when applied during the early differentiation period only. This could be due to the inhibitory effect of CLA or LA on SCD activity or to the initial activation of the differentiation factors (DMI), and the subsequent differentiation gene expression of adipogenic marker genes. It was found (36) that doses of 20–100 mmol/L LA might activate a chimera of the PPAR/human glucocorticoid receptor expressed in a cell line derived from the ovary of the Chinese hamster (36). The induction level was maximal when a dose of 150 μmol/L was applied. However, the dose of 300 μmol/L LA caused complete detachment of the cells. This indicated that the very high level of FFA could be toxic to the cells. Satory and Smith (9) reported that the inclusion of a mixture of CLA at 100 mg/L or at 356 μmol/L resulted in destruction of the 3T3-L1 cells. From our preliminary experiments, 70 mg/L of LA or CLA, especially c9, t11-CLA, was found to inhibit the proliferation of preadipocytes when supplied to media during the early proliferation period. However, viability of the cells was not affected during the differentiation period when the cell culture reached confluence (Fig. 3). This suggests that the cells under postconfluence condition could tolerate the CLA or LA dose used in the present study.

In conclusion, the present study indicated that inhibitory effects of CLA on differentiation, GPDH activity, and FA accumulation of 3T3-L1 cells are dependent on the isomer type, treatment period, and dose. t10,c12-CLA depressed the differentiation, the cellular GPDH activity, and FA accumulation in differentiating 3T3-L1 cells, whereas c9,t11-CLA and LA enhanced these under certain conditions. However, a high amount of c9,t11-CLA and LA, as well as t10,c12-CLA, also depressed the differentiation, cellular GPDH activity, and FA accumulation when they were applied during the early differentiation period.

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Fatty Acids and Monoacylglycerols Inhibit Growth of *Staphylococcus aureus*

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ABSTRACT: *Staphylococcus aureus* causes a variety of human infections including toxic shock syndrome, osteomyelitis, and mastitis. Mastitis is a common disease in the dairy cow, and *S. aureus* has been found to be a major infectious organism causing mastitis. The objectives of this research were to determine which FA and esterified forms of FA were inhibitory to growth of *S. aureus* bacteria. FA as well as their mono-, di-, and triacylglycerol forms were tested for their ability to inhibit a human toxic shock syndrome clinical isolate (MN8) and two *S. aureus* clinical bovine mastitis isolates (305 and Novel). The seven most potent inhibitors across all strains tested by minimum inhibitory concentration analysis included lauric acid, glycerol monolaurate, capric acid, myristic acid, linoleic acid, *cis*-9, *trans*-11 conjugated linoleic acid, and *trans*-10, *cis*-12 conjugated linoleic acid. Some of these lipids were chosen for 48-h growth curve analysis with a bovine mastitis *S. aureus* isolate (Novel) at doses of 0, 20, 50, and 100 μg/mL except myristic acid, which was tested at 0, 50, 100, and 200 μg/mL. The saturated FA (lauric, capric, myristic) and glycerol monolaurate behaved similarly and reduced overall growth. In contrast, the polyunsaturated FA (linoleic and *cis*-9, *trans*-11 conjugated linoleic acid) delayed the time to initiation of exponential growth in a dose-dependent fashion. The results suggest that lipids may be important in the control of *S. aureus* during an infection.

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Staphylococcus aureus is a broad host range pathogen and a leading cause of infections in both humans and domesticated animals worldwide. Infections caused by *S. aureus* are extremely common and often life threatening, creating the potential for *S. aureus* to increase morbidity and mortality. *S. aureus* causes nearly any type of human infection including toxic shock syndrome (TSS), osteomyelitis, and mastitis (1,2). *S. aureus* also causes mastitis in dairy cattle, which is the most economically important disease to the dairy industry in the United States (3). Mastitis is also a common disease during human lactation, affecting 20% to 33% of women in developed and developing countries (4,5). *S. aureus* has been found to be the main infectious organism in cases of human mastitis (6,7).

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Abbreviations: CLA, conjugated linoleic acid; GML, glycerol monolaurate; MIC, minimum inhibitory concentration; OD_{600}, optical density at 600 nm; TSB, tryptic soy broth; TSS, toxic shock syndrome.

Early reports (8,9) have characterized the ability of lipids to inhibit bacteria. Various FA and monoacylglycerols in trace amounts have an inhibitory effect on the growth of microorganisms (10). Both bacteriostatic (8,9,11) and bactericidal effects (8,12,13) have been observed. Lauric acid and glycerol monolaurate (GML) have been extensively characterized in their ability to inhibit the growth and toxin production of *S. aureus* (14,15). Other FA in milk have also been shown to inhibit bacterial growth including capric acid, myristic acid, linoleic acid, and linolenic acid. This inhibition of growth by FA has been demonstrated in several species of bacteria including *S. aureus*, *Streptococcus pneumoniae*, and *Streptococcus* group A (10,16). One of the hallmark signs of an infection caused by *S. aureus* bacteria is a release of FFA in the infected area as observed in intraperitoneal abscesses, skin infections, and mastitis across a variety of animal models (17–20). Increased concentrations of FFA in milk are common during mastitis in cows (21). Increases of 50% to 100% have been detected in FFA content of milk from cows with mastitis (22–26). However, despite the impact of mastitis on the health of the cow or woman, little is known about the effect of these FA on bacteria during an infection. To begin to investigate this, we hypothesized that certain FA and monoacylglycerols commonly found in milk would inhibit growth of *S. aureus*.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. The bacterial strains used in this study represent two different human diseases caused by *S. aureus* and include *S. aureus* MN8, a well-characterized human TSS isolate (27), and two clinical bovine mastitis isolates, *S. aureus* Novel (28) and *S. aureus* 305 (29). Cultures of each strain were prepared by inoculating a single colony into 3 mL of tryptic soy broth (TSB) (VWR, Gibbstown, NJ, USA) before incubation at 37°C overnight with shaking at 250 rpm. All minimum inhibitory concentrations (MIC) were done in duplicate and cultured in 3 mL of TSB containing a serial dilution of each particular FA. Lipids (Sigma, St. Louis, MO, USA) were solubilized in ethanol before adding to TSB to get a final concentration of 0, 12.5, 25, 50, 100, and 200 μg/mL. A control tube containing TSB and an equivalent amount of ethanol was included. Culture tubes were inoculated 1:100 with the overnight culture of the *S. aureus* strain tested and incubated 24 h with continuous shaking

at 250 rpm to determine if growth occurred. The minimum concentration necessary to prevent turbid growth was considered the MIC and evaluated visually. Growth curves were performed in triplicate and used tryptic soy broth (25 mL) inoculated 1:100 with overnight culture of *S. aureus* Novel, adjusted to an optical density at 600 nm (OD_{600}) of 0.1 units. The cultures were allowed to grow for 2 h with continuous shaking at 250 rpm to reach the growth phase. Lipids including lauric acid, GML, capric acid, myristic acid, linoleic acid, and *cis*-9, *trans*-11 conjugated linoleic acid (CLA) were then added at 0, 20, 50, and 100 μg/mL concentrations (except myristic acid, which was tested at 0, 50, 100, and 200 μg/mL) and the incubation was continued at 37°C with continuous shaking at 250 rpm. Samples were taken frequently, and the OD_{600} was read after appropriate dilution (whenever the absorbance exceeded a value of 1.0, dilutions were made until the value was within the linear range, and the absorbance value was multiplied by the dilution factor to obtain the OD_{600} value) using a Perkin-Elmer MBA2000 spectrophotometer (Boston, MA, USA). We found 1 OD_{600} unit is equivalent to 2.25 ± 0.29 mg/mL dry weight of *S. aureus* Novel.

Statistics. Minimum inhibitory concentrations were conducted twice and reported as the mean of duplicate runs. Statistical analysis of growth curves used the typical parabolic pattern of bacterial population growth. Interestingly, in some of our experimental cultures, we observed a lag phase representing delay in onset of increased growth followed by a parabolic curve. The ideal description of bacterial growth in our system, therefore, should include both the linear lag phase and the parabolic growth phase in a jointed regression, allowing estimation of the joint points and the maximum growth achieved. The fitted model was of the form:

$$Y = \beta_o + \beta_1 t + \beta_2 t^2 \text{ for } t > t_o$$
$$Y = c \text{ for } t \leq t_o$$

where Y is the OD_{600}, t is time in h, t_o is the time joint point is reached (lag before exponential growth), c is a constant representing the value of OD_{600} during the lag phase, and β_o, β_1, and β_2 are regression coefficients associated with the intercept, slope, and inflection (maximum) point, respectively. A generalized nonlinear estimated algorithm was used for parameter estimation. Separate models were estimated for each of the FA tested. The adequacy of fit was determined by the significance of the parameter estimates, and their corresponding signs and magnitudes, examination of the model residuals, and the structure of correlation among estimated parameters. A full model dummy variable regression procedure was used to conduct preplanned contrasts to compare coincidence (equality) of estimated lines, joint points, and maximum values at specific doses. Contrasts of estimated lines included all parameter estimates. Significance was declared at $P < 0.05$. Statistical computations were performed using the PROC NLMIXED procedure of SAS (30).

RESULTS

Minimum inhibitory concentrations. The MIC of the major FA present in milk along with several of their monoacylglycerols, diacylglyerols, and triacylglycerols forms were determined for two *S. aureus* bovine mastitis isolates (strain 305 and Novel), as well as the TSS isolate, MN8 (Table 1). The lipids that were the most inhibitory were similar for all three strains (except oleic acid was not inhibitory to strain MN8) and included lauric acid, glycerol monolaurate (GML), linoleic acid, *cis*-9, *trans*-11 conjugated linoleic acid (CLA), *trans*-10, *cis*-12 CLA, capric acid, and myristic acid. As shown in Table 1, the majority of FFA and their triglycerides from C6:0 to C18:0 did not inhibit growth at the maximum concentration tested (200 μg/mL) except stearic acid, which was inhibitory to the Novel strain at 200 μg/mL.

Growth curves. Several of the FA shown to have inhibitory activity were further examined in batch cultures of the *S. aureus* Novel strain including capric acid, myristic acid, lauric acid, GML, linoleic acid, and *cis*-9, *trans*-11 CLA. Growth curves were performed using concentrations of 0, 20, 50, and 100 μg/mL, with the exception of myristic acid, which was tested at 0, 50, 100, and 200 μg/mL. Parameter estimates were significant, suggesting the models were reasonable and all parameters were required. The correlation among parameters was also examined and most showed < 0.95 correlation, indicating a lack of redundancy among parameters. For each dose of FA tested, the model followed data trends (data for *cis*-9, *trans*-11 CLA presented in Fig. 1). The expected lag phase followed by a parabolic curve was observed in all doses. Residuals were examined and found to be uniform and random with no discernible pattern (data not shown). The same procedure was used for all doses of the six FA tested, with very good agreement between the predicted model and the observed data (data not shown), similar to that presented for *cis*-9, *trans*-11 CLA.

Preplanned contrasts were conducted to compare growth curves, and we found that in the case of lauric acid, capric acid, linoleic acid, and *cis*-9, *trans*-11 CLA, growth curves at each dose differed from each other. In the case of GML, 0 μg/mL was not different than 20 μg/mL; however, the growth curves for 50 and 100 μg/mL differed from 0 and 20 μg/mL. In the case of myristic acid, the 200 μg/mL myristic acid growth curve differed from the growth curves for all other doses but the other growth curves were similar. To further describe how growth curves were different, contrasts of the joint points (measure of lag in the initiation of growth) and the maximum amount of growth achieved were conducted to compare these parameters at all doses.

When free lauric acid (50 and 100 μg/mL), a well-characterized inhibitory FA, was added to the growth medium (Fig. 2, top panel), the optical density of the culture increased initially but reached a maximum well below that seen in the untreated or 20 μg/mL lauric acid-supplemented cultures. Maximum growth of bacteria was 24.7 OD units at 0 μg/mL lauric acid and was re-

TABLE 1
Minimum Inhibitory Concentration (MIC) of Lipids on Strains of *Staphylococcus aureus*[a]

Lipid[b]	MN8	305	Novel
		MIC (μg/mL)	
C6:0 Caproic acid	NI	NI	NI
C8:0 Caprylic acid	NI	NI	NI
C10:0 Capric acid	100	100	100
C12:0 Lauric acid	50	50	50
Glycerol monolaurate	50	25	50
Glycerol dilaurate	100	NI	200
Glycerol trilaurate	NI	NI	NI
C14:0 Myristic acid	100	150	200
Glycerol monomyristate	200	200	200
Glycerol trimyristate	NI	NI	NI
C16:0 Palmitic acid	NI	NI	100
Glycerol monopalmitate	NI	NI	150
Glycerol dipalmitate	NI	NI	NI
Glycerol tripalmitate	NI	NI	NI
C17:0 Margaric acid	NI	NI	NI
C18:0 Stearic acid	NI	NI	200
C18:1 *cis*-9 Oleic acid	NI	100	100
C18:1 *trans*-9 Elaidic acid	NI	NI	NI
C18:1 *trans*-11 Vaccenic acid	NI	NI	NI
C18:2 *cis*-9, *cis*-12 Linoleic acid	100	50	50
Glycerol monolinoleate	200	100	200
Glycerol trilinoleate	NI	NI	NI
C18:2 *cis*-9, *trans*-11 CLA[c]	50	25	25
C18:2 *trans*-10, *cis*-12 CLA	100	50	75

[a]Numbers reported represent the mean of duplicates. NI = no inhibition observed at any dose tested.
[b]All FA tested were in FFA form. FA were esterified at the *sn*-1 position for all monoacylglycerols and at the *sn*-1 and *sn*-3 position for diacylglycerols.
[c]CLA = conjugated linoleic acid.

duced to 8.8 OD units ($P < 0.01$) at 100 μg/mL lauric acid (Table 2). Contrasts indicated that maximum growth was different at all doses of lauric acid (Table 3). These reduced values remained constant throughout the culture duration. The 20 μg/mL lauric acid had a higher maximum growth than 0 μg/mL lauric acid.

Glycerol monolaurate supplementation (Fig. 2, bottom panel, and Table 2) resulted in both a lag in the initiation of growth and a reduction in maximum growth achieved at 100 μg/mL compared to 0 μg/mL. The maximum growth yield achieved at 20 μg/mL GML was not different than maximum growth yield at 0 μg/mL GML ($P > 0.05$, Table 3). Maximum bacterial growth was different at all other doses of GML. There was a lag in the initiation of growth (joint point) at 50 and 100 μg/mL GML compared to 0 μg/mL GML.

For capric acid (Fig. 3, top panel, and Table 4), we observed a similar growth pattern to lauric acid. At 100 μg/mL capric acid there was a lower maximal growth than 0, 20, or 50 μg/mL capric acid ($P < 0.05$, Table 3). There was no difference in maximum growth at 0, 20, or 50 μg/mL capric acid. Although there was a lag in the initiation of growth at 20 vs. 50 μg/mL capric acid ($P < 0.01$, Table 3) and 0, 20, or 50 vs. 100 μg/mL capric acid ($P < 0.05$), there was very little practical difference between the joint point estimations of 0.51, 0.52, 0.56, and −0.26 OD units for 0, 20, 50, and 100 μg/mL capric acid, respectively. Myristic acid was also tested, but at slightly higher concentrations including 0, 50, 100, and 200 μg/mL based on the higher MIC value. The results of myristic acid supplementation were similar to capric acid (Fig. 3, bottom panel, and Table 4). Maximum growth was not different at any dose. However, the time that maximum growth was achieved was different at 200 μg/mL myristic acid than all other doses. The time when growth was initiated (joint point) did not differ between 0 and 100 μg/mL myristic acid.

When *cis*-9, *trans*-11 CLA was added, there was a dose-dependent lag in the initiation of bacterial growth (Fig. 4, bottom panel). Results indicated that the lag was 3.3 h with 20 μg/mL CLA and extended to 15.5 h with 100 μg/mL CLA (Table 5). Contrasts revealed that each of the joint points were different from each other (Table 3). To determine if the effect was similar to other long-chain polyunsaturated FA tested, cultures were grown with linoleic acid supplemented at the same concentrations. A similar dose-dependent lag in the initiation of growth occurred with linoleic acid (Fig. 4, top panel, and Table 5) as was observed for *cis*-9, *trans*-11 CLA. Results indicated that the lag in growth initiation at 50 μg/mL linoleic acid was different than the joint point at 0 μg/mL linoleic acid. The lag was extended with 100 μg/mL linoleic acid to 15.2 h, which was also different from 50 μg/mL linoleic acid where growth was initiated at 8.4 h. Maximum growth was not affected by either CLA or linoleic acid.

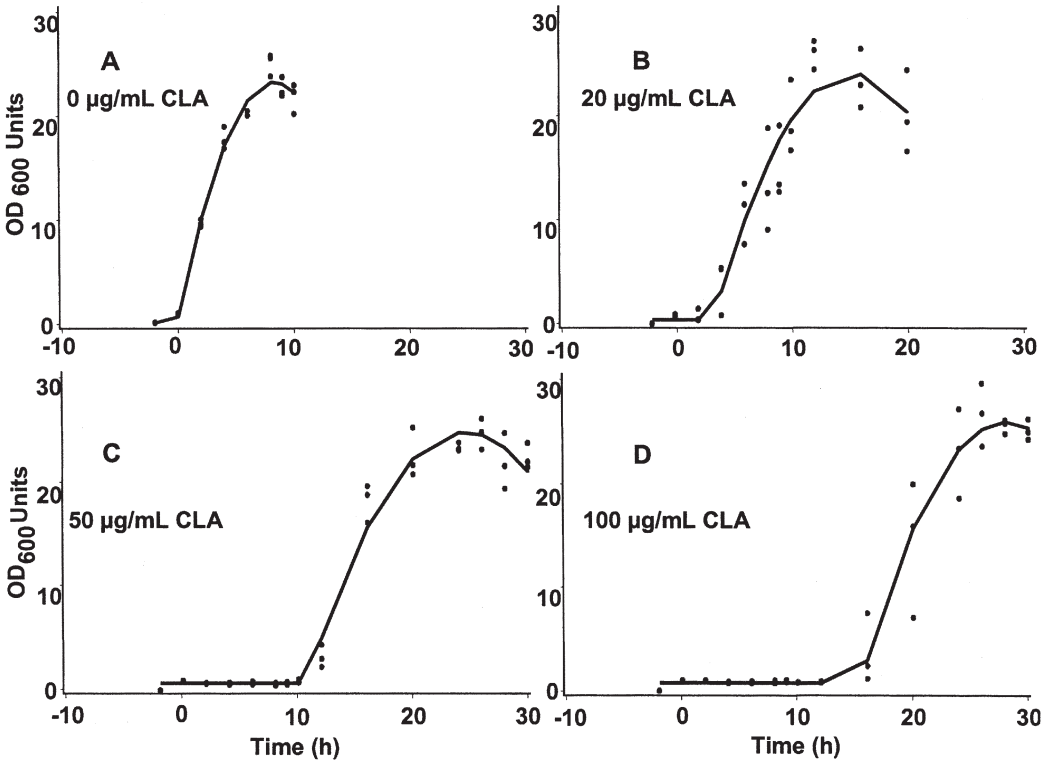

FIG. 1. Jointed nonlinear regression model fit to growth of *Staphylococcus aureus* Novel with 0 (A), 20 (B), 50 (C), or 100 µg/mL (D) of *cis*-9, *trans*-11 conjugated linoleic acid (CLA). Points are values for three replicates for each time of observation.

DISCUSSION

Fatty acids as antimicrobial agents. It has long been known that lipids have an inhibitory effect on bacteria (8,9). Trace amounts of FA have been shown to influence the growth of microorganisms in a very specific manner; certain FA such as lauric acid are more inhibitory than others (10). Kabara and coworkers (11) examined several specific straight-chain saturated FA and found lauric acid to be one of the most potent bacteriostatic FA when tested on gram-positive organisms in agreement with the present study results. They also investigated the effect of esterification and found that GML was the only monoacylglycerol more active than the FFA form, similar to our results (Table 1) where GML had the same or slightly lower MIC than lauric acid depending on the strain of *S. aureus* tested (11). The MIC of lauric acid (11) for *S. aureus* was higher (498 µg/mL) than what we observed (50 µg/mL). This may be due to differences in the sensitivity of *S. aureus* strains to certain lipids, as was found in the present study with 305 more sensitive than MN8 or Novel (Table 1). Kabara and coworkers (11) did not report what strain of *S. aureus* was used to determine their MIC (11). Our results indicate that certain FA and monoacylglycerols are more potent than others in their ability to inhibit bacterial growth similar to the previously described literature (8,9,12,13). In addition, we have demonstrated that *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA can inhibit growth of *S. aureus* (Table 1). We are not aware of previous reports on the effect of CLA on the growth of *S. aureus.*

We observed a very specific growth curve response when different FA were tested. The saturated FA, capric acid, myristic acid, and more dramatically, lauric acid, inhibited growth of bacteria through a decrease in the maximal amount of growth achieved. However, the polyunsaturated FA, linoleic, and *cis*-9, *trans*-11 CLA achieved inhibition through a dose-dependent lag in the initiation of growth. Once started, growth proceeded in a similar pattern to control. Previous reports with other species of bacteria including *Lactobacillus helveticus*, *Clostridium sporogenes,* and *Bacillus subtilis* observed similar growth inhibition with linoleic acid (10,31).

Our research suggests that some saturated and polyunsaturated FA inhibit the growth of *S. aureus* bacteria but by different mechanisms. It was also noted that in studies (10) with *Clostridium botulinum* that there may be different mechanisms of inhibition by saturated and unsaturated FA. Kodicek and Worden (32) characterized the action of unsaturated FA (i.e., linoleic acid) as bacteriostatic and reversible by surface-active agents like sterols and lecithin in *Lactobacillus* (32). Nieman (10) suggested that the inhibitory properties of FA appear more pronounced with increased chain length and degree of unsaturation (10). Knapp and Melly (12) found bactericidal effects on *S. aureus* mediated by peroxidation of arachidonic acid. Chamberlain and coworkers (33) demonstrated that addition of oleic

TABLE 2
Parameter Estimates for Growth of *Staphylococcus aureus* Novel Supplemented with Lauric Acid and Glycerol Monolaurate

		Lauric acid			Glycerol monolaurate		
Dose (μg/mL)	Parameter	Parameter estimate	Standard error	*P* value[a]	Parameter estimate	Standard error	*P* value
0	*c*	1.16	0.48	0.02	0.21	0.72	NS
	β_0	−4.54	1.35	<0.01	1.42	0.58	0.02
	β_1	8.16	0.61	<0.01	4.19	0.16	<0.01
	β_2	−0.59	0.06	<0.01	−0.21	0.01	<0.01
	Joint point[b]	0.74	0.18	0.01	−0.28	0.24	NS
	Time to max[c]	6.89	0.22	<0.01	9.96	0.15	<0.01
	Value at max[d]	24.72	0.59	<0.01	22.52	0.79	<0.01
20	*c*	1.18	0.90	NS	−0.01	0.86	NS
	β_0	−0.81	1.60	NS	1.58	0.69	0.03
	β_1	6.53	0.53	<0.01	4.21	0.19	<0.01
	β_2	−0.38	0.04	<0.01	−0.21	0.01	<0.01
	Joint point	0.31	0.27	NS	−0.37	0.26	NS
	Time to max	8.54	0.20	<0.01	10.26	0.21	<0.01
	Value at max	28.28	1.04	<0.01	23.18	0.95	<0.01
50	*c*	1.00	0.00	<0.01	0.44	0.33	NS
	β_0	1.80	0.64	<0.01	−9.90	1.40	<0.01
	β_1	3.59	0.38	<0.01	3.40	0.30	<0.01
	β_2	−0.35	0.05	<0.01	−0.15	0.01	<0.01
	Joint point	−0.22	0.19	NS	3.60	0.28	<0.01
	Time to max	5.16	0.22	<0.01	11.54	0.25	<0.01
	Value at max	12.07	0.44	<0.01	10.17	0.45	<0.01
100	*c*	1.00	0.00	<0.01	0.32	0.18	NS
	β_0	1.77	0.46	<0.01	−19.99	2.29	<0.01
	β_1	2.42	0.27	<0.01	3.62	0.35	<0.01
	β_2	−0.24	0.03	<0.01	−0.12	0.01	<0.01
	Joint point	−0.31	0.20	NS	7.43	0.23	<0.01
	Time to max	4.96	0.20	<0.01	15.08	0.20	<0.01
	Value at max	8.76	0.33	<0.01	7.67	0.36	<0.01

[a]Significance represents whether parameter estimate is different from zero. NS = not significant.
[b]Joint point = time (h) where exponential growth was initiated.
[c]Time to max = time (h) when maximal growth was achieved.
[d]Value at max = amount of growth (OD units) at the time when maximal growth was achieved.

TABLE 3
Selected List of Preplanned Contrasts of Estimated Parameters for Growth of *Staphylococcus aureus* Novel Strain with a Variety of Lipids

	Lipid				
Preplanned contrast[a]	Lauric acid	GML[b]	Capric acid	Linoleic acid	CLA[c]
			Pr > F[d]		
Joint point					
0 vs. 20 μg/mL	NS[e]	NS	NS	<0.01	<0.01
0 vs. 50 μg/mL	<0.01	<0.01	NS	<0.01	<0.01
0 vs. 100 μg/mL	<0.01	<0.01	0.02	<0.01	<0.01
20 vs. 50 μg/mL	<0.01	<0.01	<0.01	<0.01	<0.01
20 vs. 100 μg/mL	<0.01	<0.01	0.01	<0.01	<0.01
50 vs. 100 μg/mL	<0.01	<0.01	0.01	<0.01	<0.01
Max value					
0 vs. 20 μg/mL	<0.01	NS	NS	<0.01	NS
0 vs. 50 μg/mL	<0.01	<0.01	NS	<0.01	0.01
0 vs. 100 μg/mL	<0.01	<0.01	<0.01	0.02	<0.01
20 vs. 50 μg/mL	<0.01	<0.01	NS	NS	NS
20 vs. 100 μg/mL	<0.01	<0.01	<0.01	<0.01	NS
50 vs. 100 μg/mL	<0.01	<0.01	<0.01	0.01	NS

[a]Joint point = time when exponential growth was initiated; Max value = amount of growth (OD units) at the time when maximal growth was achieved.
[b]GML = glycerol monolaurate.
[c]CLA = *cis*-9, *trans*-11 conjugated linoleic acid.
[d]Pr > F = Probability of larger F.
[e]NS = not significant.

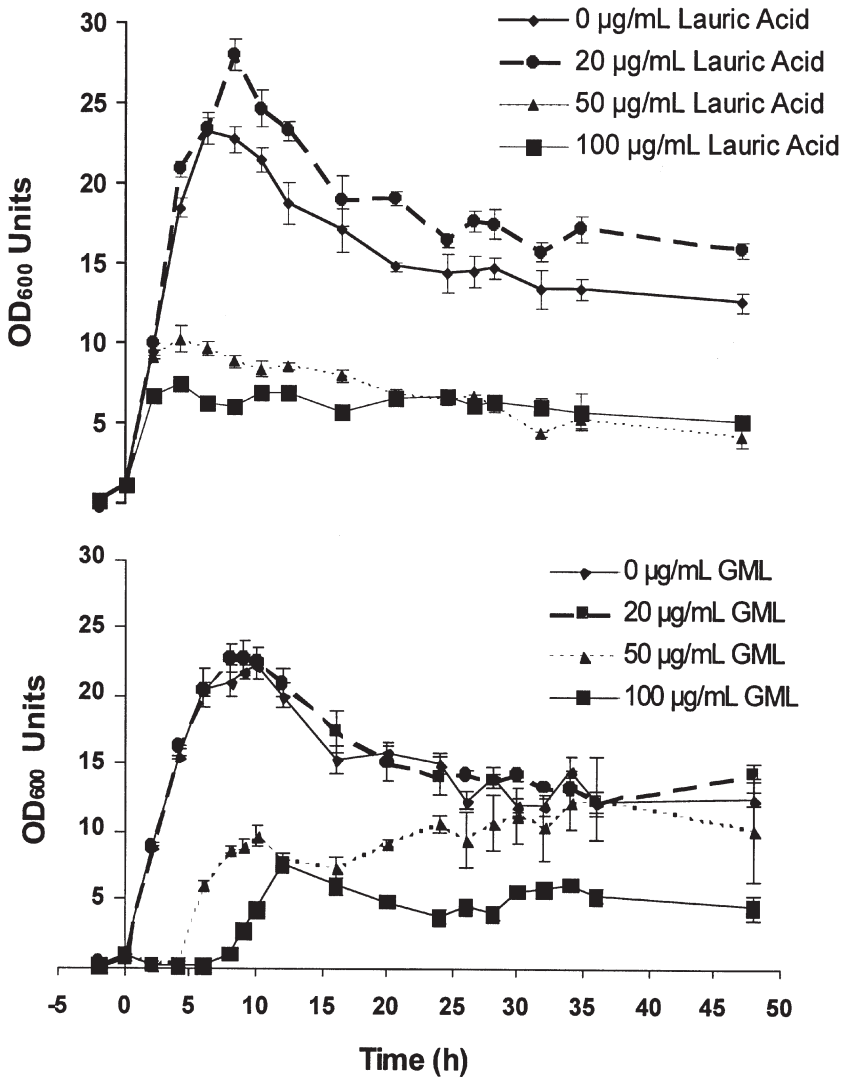

FIG. 2. Growth curves of *Staphylococcus aureus* Novel bacteria incubated with 0 (diamonds), 20 (circles), 50 (triangles), or 100 µg/mL (squares) of lauric acid (top panel) or glycerol monolaurate (bottom panel). Curves represent average of cultures performed in triplicate ± SE.

acid to the *S. aureus* resulted in increased membrane fluidity when measured by fluorescence polarimetry. This change in fluidity was detected as early as 30 s following treatment; however, significant killing occurred 45 min after treatment, indicating that oleic acid-induced killing was a multiple step process beginning with a change in fluidity of the membrane that may disrupt signal transduction cascades (33). Although proposed mechanisms (10) rely on models where the lipid interacts with the cell membrane to change permeability, more recent reports (34) indicate that some lipids including lauric acid and GML disrupt signal transduction cascades that inhibit the production of exoprotein virulence factors by *S. aureus* at concentrations lower than those required to affect growth. These observations support a multistep hypothesis to FA-induced inhibition.

FA release during infection. One indication of *S. aureus* infection is an increase in the concentration of FFA. This has been observed across a variety of infections in various animal models and in humans. Dye and Kapral (17) induced intraperitoneal abscesses in mice with *S. aureus* and found a substance produced in the abscess that was bactericidal, which was found in the lipid fraction, and further partitioning revealed it was the FFA portion. Once these FA were made into methyl esters, however, there was a loss of bactericidal activity (17). Further studies by Engler and Kapral (35) revealed a monoacylglycerol produced in intraperitoneal abscesses also had bactericidal activity. The 2-monoacylglycerol was the form originally produced; however, it spontaneously isomerized to a 1-monoacylglycerol. The FA moiety of the monoacylglycerol consisted of mostly palmitoleic acid and palmitic acid (35).

Patients with atopic dermatitis have recurring *S. aureus* skin infections and have a very different distribution of FA in their epidermis when compared to those without this condition (18). *In vitro* tests to evaluate the antimicrobial activity of human

TABLE 4
Parameter Estimates for Growth of *Staphylococcus aureus* Novel Supplemented with Capric Acid and Myristic Acid

	Capric acid				Myristic acid			
Parameter	Dose (μg/mL)	Parameter estimate	Standard error	*P* value[a]	Dose (μg/mL)	Parameter estimate	Standard error	*P* value
C	0	0.71	0.76	NS	0	0.55	1.94	NS
β_0		–2.61	1.36	NS		2.76	1.51	NS
β_1		6.61	0.44	<0.01		3.90	0.38	<0.01
β_2		–0.40	0.03	<0.01		–0.17	0.02	<0.01
Joint point[b]		0.51	0.22	0.03		–0.55	0.63	NS
Time to max[c]		8.21	0.14	<0.01		11.51	0.35	<0.01
Value at max[d]		25.24	0.88	<0.01		25.75	2.23	<0.01
C	20	0.74	0.76	NS	50	0.11	1.07	NS
β_0		–2.54	1.37	NS		1.45	0.84	NS
β_1		6.51	0.44	<0.01		3.91	0.21	<0.01
β_2		–0.36	0.03	<0.01		–0.16	0.01	<0.01
Joint point		0.52	0.22	0.02		–0.33	0.35	NS
Time to max		8.91	0.21	<0.01		12.22	0.23	<0.01
Value at max		27.21	0.88	<0.01		25.49	1.23	<0.01
C	50	0.58	0.79	NS	100	0.07	0.94	NS
β_0		–2.51	1.09	0.02		2.24	0.74	<0.01
β_1		5.72	0.28	<0.01		3.86	0.19	<0.01
β_2		–0.28	0.01	<0.01		–0.16	0.01	<0.01
Joint point		0.56	0.23	0.02		–0.55	0.31	NS
Time to max		10.07	0.13	<0.01		12.27	0.21	<0.01
Value at max		26.89	0.89	<0.01		26.05	1.08	<0.01
c	100	1.00	0.00	<0.01	200	1.00	<0.01	<0.01
β_0		1.74	0.85	0.04		3.95	1.04	<0.01
β_1		2.81	0.25	<0.01		1.35	0.20	<0.01
β_2		–0.11	0.02	<0.01		–0.02	<0.01	<0.01
Joint point		–0.26	0.31	NS		–2.1	0.95	0.03
Time to max		13.07	0.81	<0.01		26.91	3.79	<0.01
Value at max		21.14	0.57	<0.01		23.10	1.10	<0.01

[a]Significance represents whether parameter estimate is different from zero. NS = not significant.
[b]Joint point = time (h) where exponential growth was initiated.
[c]Time to max = time (h) when maximal growth was achieved.
[d]Value at max = amount of growth (OD units) at the time when maximal growth was achieved.

stratum corneum lipids against *S. aureus* have only been done with extracts from the skin of healthy individuals. However, the FFA fraction has been found to be the most effective class of lipid against growth of *S. aureus* (19). Further studies found lauric acid and linoleic acid to be the most active of these FFA, similar to the present study results (20).

The concentrations of FA tested in the present study were well within the normal range found in bovine and human milk. The profiles of bovine and human milk are slightly different, with bovine milk containing a higher content of short-chain saturated FA and human milk containing a higher concentration of unsaturated long-chain FA (36). Increased concentrations of FFA in milk are common during mastitis in cows (21). Increases of 50% to 100% have been detected in FFA content of milk from cows with mastitis (22–26). The concentrations of individual FFA during mastitis ranged from approximately 17 to 312 μg/mL with lauric acid and linoleic acid present at 50 and 56 μg/mL (an increase of 20% and 60% over normal concentrations), respectively (37). The increase in FFA during infection of the mammary gland was not constant among all FA (4.7% increase in capric acid vs. 77.2% increase in stearic acid), presumably due to the amount of that particular FA present in milk fat and their preferential position on the triglyceride molecule. Preference for butyric acid, caproic acid, and caprylic acid is at the *sn*-3 position, while lauric acid, myristic acid, and palmitic acid are typically located at the *sn*-2 position (36). However, lipases secreted by *S. aureus* have no positional specificity and hydrolyze FFA at all positions on the triglyceride equally (38). Nair *et al.* (39) tested the antibacterial effect of caprylic acid and monocaprylin and showed these lipids were able to kill five different mastitis pathogens in milk. However, the doses used were ~7,200 or ~14,000 μg/mL caprylic acid and ~5,500 or ~11,000 μg/mL monocaprylin, well above the range of those lipids present even in the triglyceride fraction of milk. The doses of FA used in the present study (20, 50, 100, and 200 μg/mL) are within the physiological range of those specific FA released during a mastitis infection.

Impact on host/pathogen interaction. FA at lower concen-

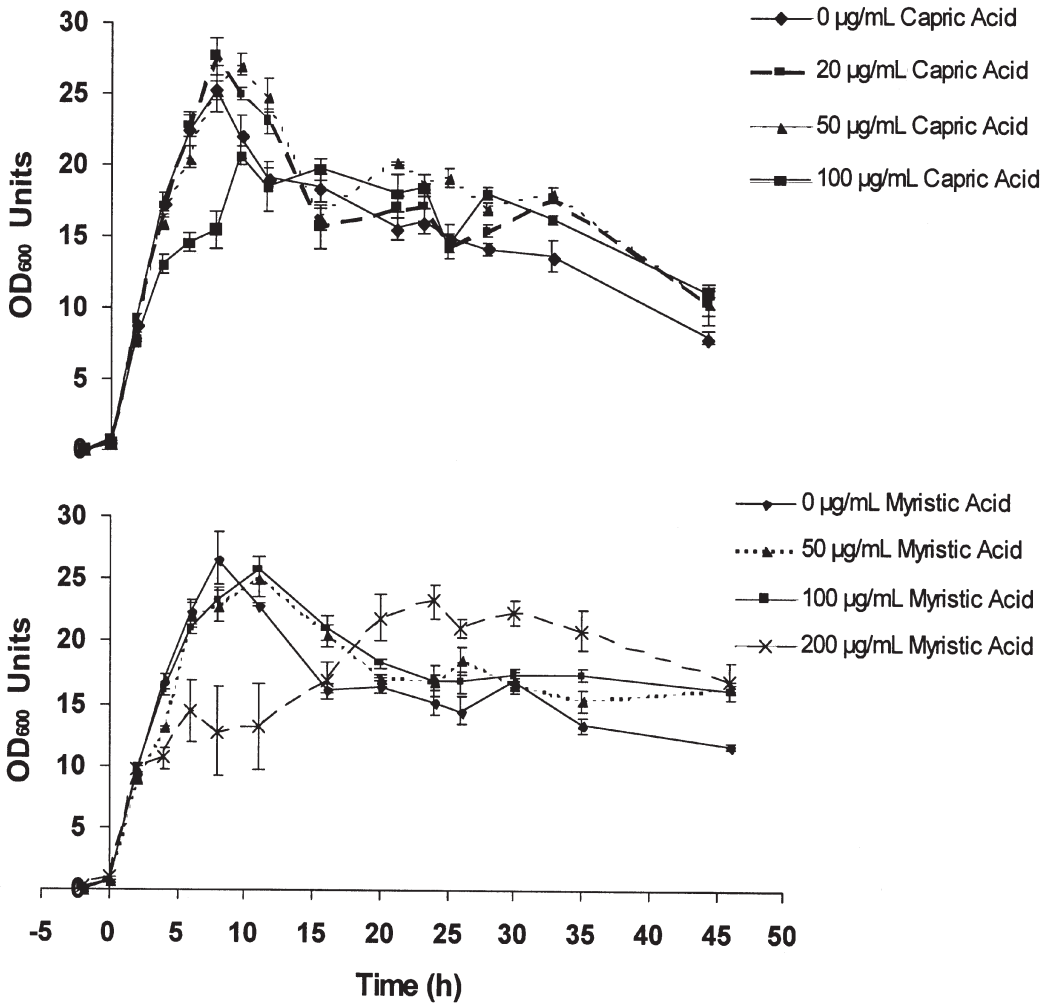

FIG. 3. Growth curves of *Staphylococcus aureus* Novel bacteria incubated with 0 (diamonds), 20 (circles), 50 (triangles), or 100 µg/mL (squares) of capric acid (top panel) or 0 (diamonds), 50 (triangles), 100 (squares), or 200 µg/mL (crosses) of myristic acid (bottom panel). Curves represent average of cultures performed in triplicate ± SE.

trations than those required for growth inhibition may also have other effects on the bacteria that are beneficial to the host. Lauric acid and GML have been more specifically analyzed in their ability to inhibit the growth and toxin production of *S. aureus*. Schlievert and coworkers (14) demonstrated the ability of GML to reduce the production of exotoxins by *S. aureus* at concentrations lower than those required for growth inhibition. The growth curves for the bovine mastitis *S. aureus* isolates followed a similar pattern to growth curves presented by Schlievert *et al.* (14) with human TSS isolates. Further work by Ruzin and Novick (15) found that free lauric acid at equimolar concentrations was just as effective as GML in blocking expression of protein A and TSS toxin-1 in *S. aureus.* Importantly, they found that active lipases secreted by *S. aureus* rapidly hydrolyze lauric acid from GML. This inhibition by lauric acid was at a dose of 20 µg/mL, a concentration 50-fold lower than that found in milk. Projan *et al.* (34) determined that GML inhibition of staphylococcal toxins occurred at the level of transcription. They also demonstrated that reduction of virulence factors was independent of the *agr* virulence regulatory system. Recently, GML was also shown to reduce three anthrax toxin components at the transcriptional level (40). We observed a difference in the pattern of growth inhibition between lauric acid (Fig. 2, top panel) and GML (Fig. 2, bottom panel). Lauric acid inhibited the maximum growth achieved by the bacteria whereas GML inhibited the growth of *S. aureus* through both a delay in the initiation of growth and a decrease in the maximum growth achieved. We speculate that this difference might be attributed to the amount of time it takes *S. aureus* to secrete active lipases able to cleave free lauric acid from the glycerol backbone of GML. Initially, GML inhibits the growth of *S. aureus* similarly to the polyunsaturated long-chain FA, creating a dose-dependent lag in the initiation of exponential growth. However, after 12 h, the pattern of growth is similar to that seen for lauric acid. Expression of lipases occurs normally during the postexponential phase of growth (41,42). However,

TABLE 5
Parameter Estimates for Growth of *Staphylococcus aureus* Novel Supplemented with *cis*-9, *trans*-11 Conjugated Linoleic Acid and Linoleic Acid

		cis-9, *trans*-11 CLA			Linoleic acid		
Dose (μg/mL)	Parameter	Parameter estimate	Standard error	*P* value[a]	Parameter estimate	Standard error	*P* value
0	*c*	0.13	0.64	NS	0.64	0.56	NS
	β_0	0.68	0.57	NS	−5.36	1.43	<0.01
	β_1	5.43	0.27	<0.01	7.53	0.47	<0.01
	β_2	−0.32	0.02	<0.01	−0.45	0.03	<0.01
	Joint point[b]	−0.03	0.20	NS	0.84	0.18	<0.01
	Time to max[c]	8.29	0.27	<0.01	8.28	0.14	<0.01
	Value at max[d]	23.35	0.70	<0.01	26.52	0.74	<0.01
20	*c*	0.47	0.94	NS	0.99	0.56	NS
	β_0	−14.28	3.15	<0.01	−38.55	5.34	<0.01
	β_1	5.05	0.58	<0.01	10.58	1.03	<0.01
	β_2	−0.16	0.02	<0.01	−0.40	0.05	<0.01
	Joint point	3.26	0.46	<0.01	4.51	0.26	<0.01
	Time to max	15.23	0.57	<0.01	13.14	0.30	<0.01
	Value at max	24.71	1.28	<0.01	31.98	0.92	<0.01
50	*c*	0.75	0.28	0.01	0.98	0.29	<0.01
	β_0	−51.09	4.30	<0.01	−52.88	4.35	<0.01
	β_1	6.19	0.43	<0.01	8.12	0.55	<0.01
	β_2	−0.12	0.01	<0.01	−0.20	0.02	<0.01
	Joint point	10.71	0.28	<0.01	8.35	0.21	<0.01
	Time to max	24.52	0.34	<0.01	20.26	0.31	<0.01
	Value at max	25.59	0.51	<0.01	30.42	0.55	<0.01
100	*c*	0.92	0.38	0.02	0.87	0.24	<0.01
	β_0	−98.05	12.64	<0.01	−139.24	10.14	<0.01
	β_1	8.83	1.13	<0.01	13.19	0.95	<0.01
	β_2	−0.15	0.02	<0.01	−0.26	0.02	<0.01
	Joint point	15.46	0.33	<0.01	15.16	0.17	<0.01
	Time to max	28.08	0.86	<0.01	25.29	0.31	<0.01
	Value at max	26.90	0.73	<0.01	28.52	0.46	<0.01

[a]Significance represents whether parameter estimate is different from zero. NS = not significant.
[b]Joint point = time (h) where exponential growth was initiated.
[c]Time to max = time (h) when maximal growth was achieved.
[d]Value at max = amount of growth (OD units) at the time when maximal growth was achieved.

some exoproteins including lipases may have individual specific regulatory mechanisms that may be induced by their substrates (41). The effect on *S. aureus* toxin production has been observed in lauric acid and GML; however, we are unaware of any reports in which these effects have been observed with polyunsaturated long-chain FA.

These results indicate that release of FFA or monoacylglycerols may be a mechanism to inhibit growth of *S. aureus* during an infection. At subinhibitory concentrations, these FA may also help to minimize damage to host tissues due to virulence factors such as toxins secreted by *S. aureus.*

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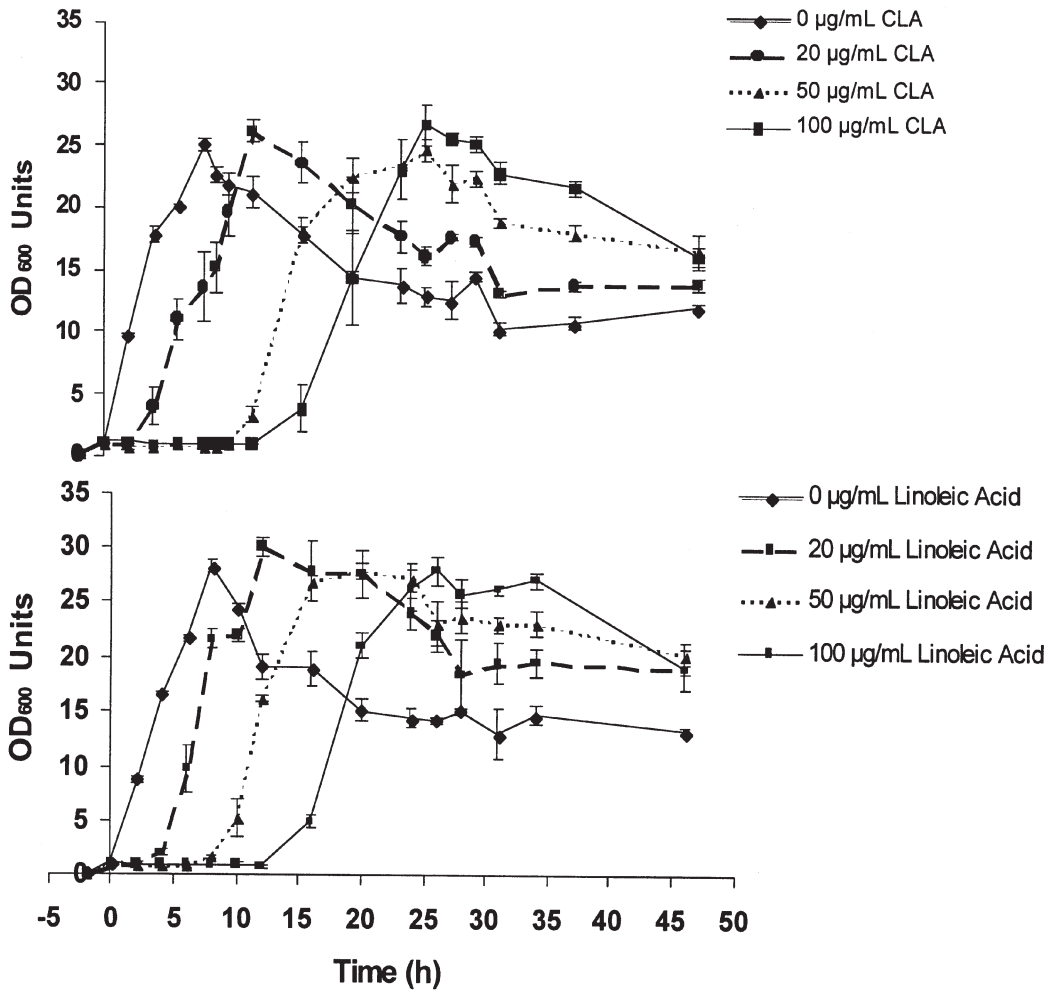

FIG. 4. Growth curves of *Staphylococcus aureus* Novel bacteria incubated with 0 (diamonds), 20 (circles), 50 (triangles), or 100 µg/mL (squares) of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (top panel) or linoleic acid (bottom panel). Curves represent average of cultures performed in triplicate ± SE.

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REVIEW

Dietary *trans* Fatty Acids: Review of Recent Human Studies and Food Industry Responses

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ABSTRACT: Dietary *trans* FA at sufficiently high levels have been found to increase low density lipoprotein (LDL)-cholesterol and decrease high density lipoprotein (HDL)-cholesterol (and thus to increase the ratio of LDL-cholesterol/HDL-cholesterol) compared with diets high in *cis* monounsaturated FA or PUFA. The dietary levels of *trans* FA at which these effects are easily measured are around 4% of energy or higher to increase LDL-cholesterol and around 5 to 6% of energy or higher to decrease HDL-cholesterol, compared with essentially *trans*-free control diets. Very limited data at lower levels of intake (less than 4% of energy) are available. Most health professional organizations and some governments now recommend reduced consumption of foods containing *trans* FA, and effective January 1, 2006, the U.S. Food and Drug Administration requires the labeling of the amounts of *trans* FA per serving in packaged foods. In response, the food industry is working on ways to eliminate or greatly reduce *trans* FA in food products. Current efforts focus on four technological options: (i) modification of the hydrogenation process, (ii) use of interesterification, (iii) use of fractions high in solids from natural oils, and (iv) use of trait-enhanced oils. Challenges to the food industry in replacing *trans* FA in foods are to develop formulation options that provide equivalent functionality, are economically feasible, and do not greatly increase saturated FA content.

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In recent years, dietary *trans* FA have raised health concerns largely from reports that high levels in the diet, compared with high levels of *cis* FA, have resulted in unfavorable effects on both low density lipoprotein (LDL)- and high density (HDL)-cholesterol. In response, most health professional organizations have recommended reduced consumption of foods containing *trans* FA, and the U.S. Food and Drug Administration (FDA) has issued regulations requiring the labeling of *trans* FA on packaged foods effective January 1, 2006. In addition, many food manufacturers who have used partially hydrogenated oils in their products have developed or are considering ways to reduce or eliminate *trans* FA from these products.

This review covers the following topics: (i) occurrence of *trans* FA in the U.S. food supply; (ii) review of human studies relating dietary *trans* FA to risk of coronary heart disease (CHD); (iii) review of studies relating dietary *trans* FA to other health conditions: cancer, maternal and child health, type 2 diabetes, and macular degeneration; (iv) dietary recommendations regarding *trans* and saturated FA by health professional organizations; and (v) recent food industry efforts to replace or reduce *trans* FA in foods. The possible relationship between dietary factors, such as *trans* and saturated FA, and CHD is important because CHD is the most common form of cardiovascular disease in the industrialized world. Key risk factors for CHD include positive family history (genetics), tobacco smoking, hypertension, diabetes, and elevated serum cholesterol.

OCCURRENCE OF TRANS FA IN THE U.S. FOOD SUPPLY

Unsaturated FA in foods can exist in either the *cis* or *trans* configuration (Scheme 1). In the *cis* form, the hydrogen atoms are on the same side of the double bond. In the *trans* form they are opposite. As a result of these orientations around the double bond, the *cis* FA has a bend in the carbon chain, whereas the *trans* FA has a straight carbon chain resembling that of a saturated FA. The term "positional isomer" is commonly used to refer to *cis* or *trans* FA if one or more of the double bonds have migrated to a new position in the FA chain. *Trans* FA (with double bonds at various positions in the FA chain) and *cis* positional isomers are formed during partial hydrogenation of fats and oils, a process used to impart desirable stability and physical properties to such food products as margarines and spreads, shortenings, frying fats, and specialty fats (e.g., for fillings, toppings, and candy). In addition, small amounts of *trans* FA occur naturally in foods such as milk, butter, and tallow as a result of biohydrogenation in ruminants.

Widespread use of partially hydrogenated vegetable oils in the United States during the past four or five decades has raised questions about possible health effects resulting from the consumption of *trans* FA present in these products. Among isomeric FA, recent interest has focused on *trans* FA rather than on positional isomers of *cis* FA. Accordingly, this review deals with effects of *trans* FA rather than positional isomers of *cis* FA.

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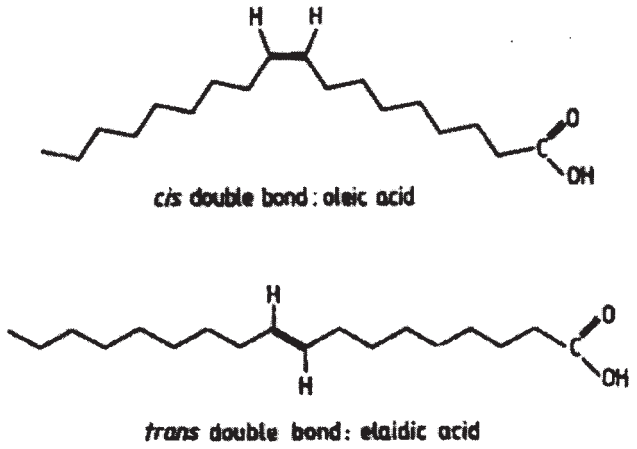
Abbreviations: AHA, American Heart Association; AMD, age-related macular degeneration; apoB-100, apolipoprotein B-100; CHD, coronary heart disease; CRP, C-reactive protein; DHA, docosahexaenoic acid; FASEB, Federation of American Societies for Experimental Biology; EFA, essential fatty acids; EPA, eicosapentaenoic acid; FDA, U.S. Food and Drug Administration; FFA, free fatty acids; FMD, flow-mediated vasodilation; HDL, high-density lipoprotein; IL, interleukin; IOM, Institute of Medicine (of the National Academy of Sciences); LDL, low-density lipoprotein; Lp(a), lipoprotein(a); sTNF-R1 and -R2, soluble tumor necrosis factor alpha receptors 1 and 2; USB, United Soybean Board; USDA, United States Department of Agriculture; WHO/FAO, World Health Organization/Food and Agricultural Organization (of the United Nations).



SCHEME 1

Typical levels of *trans* FA in food products containing partially hydrogenated oils are shown in Table 1. Frying oils used by restaurants and food service operations range in *trans* FA content from zero to about 35% of total FA. Some restaurants and food service operations currently use unhydrogenated oils (such as those used as salad oils) for frying, and such oils are *trans*-free. Until a few years ago, the fats used in the manufacture of retail margarines and spreads contained, on average, about 15–25% *trans* FA. Currently, most tub, liquid, and spray products contain no *trans* fat. The total fat content of many retail spreads, both tub and stick varieties, now averages about 55–60%. Thus, these spread products contain between 0 and 15% *trans* FA by weight. True margarines contain at least 80% fat (required by the FDA's standard of identity for margarines), but such products now represent only a small market share of the category of margarines and spreads. Most currently available baking shortenings typically contain about 15–30% *trans* FA, expressed as a percentage of total FA. In 2005, at least one marketed shortening was *trans*-free. Beef and dairy fat typically contain about 3% *trans* FA, also expressed as a percentage of total FA.

With regard to levels of consumption, Allison *et al.* (1) reported a mean intake of *trans* FA by the U.S. population of 2.6% of energy, or 5.3 g/person/d. This estimate was based on 24-h recalls and 2-d food records by over 11,200 subjects as part of the USDA's Continuing Survey of Food Intakes by Individuals (cited in Ref. 1). Consistent with these results, Harnack *et al*. (2) reported that the mean intake of *trans* FA in a population of adult subjects in the Minneapolis-St. Paul, Minnesota, USA, metropolitan area decreased from 3.0% of total energy in 1980–1982 to 2.2% of total energy in 1995–1997. The estimates of Harnack *et al.* were based on 24-h dietary recalls by more than 7,900 subjects participating in the Minnesota Heart Study, an ongoing observational epidemiologic study. These estimated intakes (1,2) of *trans* FA of 2–3% of total energy are smaller than those of saturated FA, which contribute 12–14% of energy intake (3–6). The intake of *trans* FA in 14 European countries has been reported to range from 0.5 to 2.1% of energy (7), somewhat less than the intake reported for the United States. This intake of *trans* FA in Europe also is considerably less than the intake of saturated FA in Europe, estimated to be 10–19% of energy (7).

TABLE 1
Typical Levels[a] of *trans* FA in Food Fats/Products

Food fat/product	*trans* FA content
Frying fats	0–35% of FA
Margarines/spreads	Fat: 0–25% of FA product: 0–15% by weight
Shortenings	0–30% of FA
Beef and dairy fat	3% of FA

[a]The levels of *trans* FA in various food products are expressed as a percentage of total FA.

Estimates of intake of *trans* FA based on reported consumption of foods (1,2,7) are considerably lower than estimates based on the availability from foods. The latter estimates consider market size, market share, and composition of products made from partially hydrogenated fats and oils and thus represent reasonable approximations of intake. Such estimates, however, do not take into account fats that are wasted or deliberately discarded. In 1986, Hunter and Applewhite (8) reported an estimate of *trans* FA available for consumption in the U.S. diet for 1984 of 7.6 g/person/d. A similar value, 8.3 g/person/d, was obtained independently by a Federation of American Societies for Experimental Biology (FASEB) Review Panel on *Trans* FA (9). Hunter and Applewhite (10) updated their estimate of *trans* FA availability in the U.S. diet for 1989 to be 8.1 g/person/d. Biomarker data are needed to assess *trans* FA intake more accurately. Current efforts by food manufacturers to eliminate or drastically reduce levels of *trans* FA in their products (to be discussed later in this review) are expected to reduce the intake of *trans* FA to levels well below those reported to date.

HUMAN STUDIES RELATING DIETARY *TRANS* FA TO RISK OF CHD

Review articles. (i) Reviews of studies reported prior to 1990. The year 1990 marked a period of renewed interest by many health professionals in the effects of dietary *trans* FA. That year, a study by Mensink and Katan (11) reported that high levels of *trans* FA in the diet increased the level of LDL-cholesterol and decreased the level of HDL-cholesterol compared with high levels of *cis* FA. Details of this study will be discussed subsequently.

Prior to the study by Mensink and Katan (11), numerous reviews and comprehensive studies showed that diets high in 18-carbon *trans* monoenes but adequate in EFA were not uniquely atherogenic (12–18). Many of these reviews and studies were considered in a comprehensive literature review by a special FASEB committee (9). This report concluded that there is "little reason for concern with the safety of dietary *trans* FA *both* at their present and expected levels of consumption *and* at the present and expected levels of consumption of dietary linoleic acid. . . ." A 1987 British Nutrition Foundation report on the safety of *trans* FA reached the same conclusion (19).

(ii) Reviews of studies reported since 1990. Reviews published after 1990 discussed the Mensink and Katan study (11)

and subsequent studies, suggesting that high dietary levels of *trans* FA increased CHD risk. A 1995 review (3) by Kris-Etherton and Nicolosi (before much of the *trans* fat literature was published) suggested that the association between *trans* FA intake and CHD risk is weak and inconsistent compared with the large body of evidence from epidemiologic observations and animal and human studies that supports a direct effect of saturated fat on CHD risk. These authors commented that some clinical studies were difficult to interpret because it was unclear whether the reported responses were due to the addition of *trans* FA to the diet or to a decrease in dietary unsaturated (i.e., cholesterol-reducing) FA. Kris-Etherton and Nicolosi (3) considered the current level of intake of *trans* FA to be safe; however, they supported the reduction of total fat intake (including reduced intake of *trans* and cholesterol-raising saturated FA) as part of national dietary guidelines to promote health and well-being.

Katan *et al*. (20) concluded that the effects of *trans* FA on raising plasma LDL-cholesterol levels were similar to those of saturated FA. These authors recommended that diets aimed at reducing the risk of CHD be low in both *trans* and saturated FA.

A comprehensive review by the American Society for Clinical Nutrition/American Institute of Nutrition Task Force on *trans* Fatty Acids (4) concluded that "compared with saturated FA, the issue of *trans* FA is less significant because the U.S. diet provides a smaller proportion of *trans* FA and the data on their biological effects are limited." The Task Force recommended that data be obtained that are comparable to those of saturated FA on the intake of *trans* FA, their biological effects and associated mechanisms of action, and their relation to disease.

Zock and Katan (21) reviewed 20 dietary trials comparing effects of butter and margarine on blood lipids. The authors reported that replacing butter by soft, low-*trans* margarines favorably affected the blood lipoprotein profile, which in turn might reduce the risk of CHD. Replacing butter by hard, high-*trans* margarines appeared to offer no benefit over butter.

Ascherio *et al*. (22) reported a linear relationship between the change in LDL/HDL ratio (a measure of CHD risk) and the percentage of energy from either *trans* FA or saturated FA in the diet. These authors also concluded that the adverse effect on LDL/HDL ratio of *trans* FA appeared to be stronger than that of saturated FA.

A subsequent review by Katan (23) indicated that since around 1990, *trans* FA went from a type of fat generally regarded as safe and possibly beneficial to one considered by many to be unfavorable for CHD risk. Katan felt that *trans* FA were at least as unfavorable as cholesterol-raising saturated FA. Wolfram (24) indicated that epidemiological studies have noted a strong association between intake of saturated and *trans* FA and plasma cholesterol levels and rates of CHD mortality worldwide.

A 2002 review by Schaefer (25) suggested that hydrogenated margarines and butter should be avoided, and that either oil or soft margarine low in *trans* FA should be used as a spread. Schaefer noted that in addition to having unfavorable effects on LDL- and HDL-cholesterol concentrations, there is evidence that increased *trans* FA consumption, in contrast with consumption of saturated fat, increases plasma concentrations of lipoprotein(a) [Lp(a)].

Mensink *et al*. (26) reported a meta-analysis of 60 controlled trials focusing on the effect of individual dietary FA on the ratio of total cholesterol to HDL cholesterol and on serum lipoproteins. These authors determined that this ratio was decreased most effectively (corresponding to decreased risk of CHD) when *trans* FA and saturated FA were replaced with *cis* unsaturated FA.

In contrast to the preceding reviews, Wilson *et al*. (27) concluded that the intake of *trans* FA does not pose as much of a health concern for Americans as does the intake of cholesterol-raising saturated FA. Although Wilson *et al.* encouraged efforts to minimize increased amounts of *trans* FA in the diet, they felt that *trans* FA at the current level of intake are a safe component of the diet. Wilson *et al*. suggested that consumers limit their intake of *trans* FA by lowering their intake of total fat within the context of a varied and healthful diet.

Similarly, a brief review by Steinhart *et al*. (28) noted that only a slight correlation between *trans* FA and CHD can be observed if *trans* FA are consumed instead of saturated FA. Nevertheless, the *trans* FA content of German margarines, cooking fats, and shortenings was reported to have decreased from 1992 to 1997, and estimated daily intakes of *trans* FA in Germany also decreased during this period.

The Institute of Medicine (IOM) of the National Academy of Sciences (29) reviewed effects of dietary *trans* FA as part of a report on reference intake levels of macronutrients. Citing the study of Ascherio *et al*. (22), the IOM report stated that, similar to saturated FA, there is a positive linear trend between *trans* FA intake and increased risk of CHD. The IOM report, however, did not establish a "Tolerable Upper Intake Level" above which long-term consumption might be undesirable for some individuals. The IOM noted that *trans* FA are unavoidable in ordinary diets and that eliminating them from the diet might result in inadequate intakes of certain nutrients and increase certain health risks. The report recommended that consumption of *trans* FA as well as saturated FA and cholesterol be as low as possible from a nutritionally adequate diet.

The most recent (2006) review, by Mozaffarian *et al*. (30), considers evidence for physiological and cellular effects of *trans* FA as well as the feasibility and potential implications of reducing or eliminating the consumption of *trans* FA in the United States. Consistent with other reviews, this article conveys risks associated with consumption of high levels of *trans* FA. In contrast to other reviews, the authors state that even low levels of consumption (1 to 3% of total energy intake) confer "a substantially increased risk . . . ." However, the references cited to support this statement were epidemiological and prospective studies and not controlled dietary trials in which the investigators fed *trans* FA at such levels. A controlled study by Lichtenstein *et al*. (31) found no significant effect on either LDL- or HDL-cholesterol when a diet containing *trans* FA at 3.3% of energy was fed for 35 d and compared with a control

diet with *trans* FA at only 0.6% of energy. Mozaffarian *et al*. expressed their view that consumption of *trans* FA at less than 0.5% of total energy may be necessary to avoid adverse effects. This would mean complete or near-complete avoidance of dietary *trans* FA, an extreme position compared with that expressed in the IOM report (29) and probably unlikely in the near future to meet with significant consumer acceptance.

Human trials reporting changes in blood lipid and lipoprotein levels with dietary trans FA. (i) Experimental studies. Prior to 1990, two comprehensive literature reviews (9,19) had concluded that there was little basis for concern about the consumption of *trans* FA at present and expected dietary levels. One study (of many) on which this conclusion was based was that of Mattson *et al*. (32). This study was particularly significant because it was the first to control carefully the FA composition of the diets. In this study, subjects were fed liquid formula diets high in *cis* or *trans* FA for 28 d. The FA intakes of both groups were the same except for the presence or absence of *trans* FA. The key result was that the subjects fed the high-*trans* diet had the same blood total cholesterol and TG levels as subjects fed the high-*cis* diet. At the time of this study, methodology for measuring HDL- and LDL-cholesterol and their apoproteins was not well established.

In a subsequent study by Mensink and Katan (11), blood lipoprotein levels were measured in young adult human subjects (mean age 26 yr) fed conventional food diets high in either *cis*, *trans*, or saturated FA. The study design resembled that used by Mattson *et al*. (32) in that the diets containing *cis* and *trans* FA had similar FA compositions except for the presence or absence of *trans* FA. In this study, the high-*trans* diet (10.9% of energy as *trans* FA) raised total and LDL-cholesterol and lowered HDL-cholesterol compared with the high-*cis* diet after 3 wk. Although the level of *trans* FA in the high-*trans* diet was unrealistically high (nearly 11% of energy), the authors concluded that the effect of *trans* FA on serum lipoprotein profiles was similar to that of cholesterol-raising saturated FA.

Since the Mensink and Katan study (11), numerous other human trials have been conducted that include measurement of LDL- and HDL-cholesterol [and occasionally Lp(a)] after feeding diets containing moderate or high levels of *trans* FA (31,33–61). Key study design parameters and results are summarized in Table 2. In essence, these studies demonstrated that diets high in *trans* FA raised LDL-cholesterol and lowered HDL-cholesterol compared with diets low in *trans* FA.

Three noteworthy studies (34,37,39) are discussed in more detail below. Zock and Katan (34) compared effects on blood lipoproteins of diets high in linoleic acid, *trans* FA, and stearic acid. In this study, the intake of *trans* FA was lower than that of the Mensink and Katan (11) study (7.7% of energy vs. 10.9% of energy). The lower level of *trans* FA raised total cholesterol and LDL-cholesterol and lowered HDL-cholesterol compared with the linoleic acid diet. The stearic acid diet similarly raised total and LDL-cholesterol and reduced HDL-cholesterol compared with the linoleic acid diet. Subsequently, Mensink *et al*. (33) reported that this *trans* FA diet raised Lp(a) levels compared with the linoleic acid and stearic acid diets.

In the first study by Judd *et al*. (37), moderate- and high-*trans* diets (3.8 and 6.6% of energy, respectively) resulted in increased plasma LDL-cholesterol levels compared with an oleic acid diet. These increases, however, were less than those observed with a saturated FA diet. The high-*trans* diet, but not the moderate-*trans* diet, resulted in a minor (statistically significant) reduction in HDL-cholesterol. The saturated diet, however, led to a slight increase in HDL-cholesterol.

Lp(a) levels were reported in a subsequent publication (40). Compared with the oleic diet, the *trans* diets had no effect on Lp(a) levels when all subjects were considered collectively. However, a subset with initially high levels of Lp(a) (≥30 mg/dL) responded to the high-*trans* diet with a slight (5%) increase in Lp(a) levels relative to the oleic and moderate-*trans* diets. Thus, the possibly unfavorable effects on Lp(a) of replacing *cis* with *trans* FA were restricted to high-*trans* intakes in subjects with high levels of Lp(a).

The second study by Judd *et al*. (39) included a high-carbohydrate diet to assess whether direct addition of *trans* FA to the diet had an independent cholesterol-raising effect. Also, a high-stearate diet was included to determine whether stearate and *trans* FA were neutral with regard to their effects on blood lipoproteins. Compared with a high-oleic diet, the high-*trans* (8% of energy) diet raised LDL-cholesterol to a greater extent than the high-saturated diet despite similar levels of *trans* plus saturated FA. In contrast, in the previous study (37), the increase in LDL-cholesterol after the high-*trans* diet was directionally (but not significantly) less than that observed after the high-saturated diet. Compared with the high-carbohydrate diet, both the moderate- (4% of energy) and high-*trans* diets and the saturated diet resulted in increased LDL-cholesterol levels. Both *trans* diets lowered HDL-cholesterol compared with the oleic diet but not compared with the carbohydrate diet. Also compared with the carbohydrate diet, the stearic acid diet had no effect on LDL-cholesterol but lowered HDL-cholesterol. The oleic acid diet had no effect on LDL-cholesterol but raised HDL-cholesterol.

The studies by Mensink and Katan (11), Zock and Katan (34), and Judd *et al*. (37,39) were consistent in that in all four studies, the *trans* FA and saturated FA treatments raised LDL-cholesterol levels compared with the control diet. The changes in LDL-cholesterol levels by *trans* FA and by saturated FA were similar. HDL-cholesterol levels in these studies were reduced consistently when the level of *trans* FA in the diet was 6.6% of energy or higher. In the second study by Judd and colleagues (39), the moderate-*trans* diet showed a reduction in HDL-cholesterol that was not seen in the first study (37).

(ii) Effect of dietary trans FA on LDL particle size. Mauger *et al*. (62), in an extension of the study by Lichtenstein *et al*. (31), reported that increasing the amounts of dietary *trans* FA resulted in decreased LDL particle size in a dose-dependent fashion. Mauger *et al*. cited studies reporting that small and dense LDL particles have been reported to be more atherogenic than larger, less dense particles. In contrast, Kim and Campos (63), in an epidemiological study of a Costa Rican population, found an association between intake of *trans* FA and large LDL

TABLE 2
Human Trials Reporting Changes in LDL-Cholesterol, HDL-Cholesterol, and Lipoprotein(a) with Dietary *trans* FA (TFA)

Reference	Study population	Period (d)	TFA (% en) Treatment and control diets	Change in LDL-C vs. control[a]	Change in HDL-C vs control[a]	Change in Lp(a) vs. control[a]
Mensink and Katan (11); Mensink *et al.* (33)	34 Women 25 Men Normocholesterolemic	21	10.9	13.9% increase (2.67 to 3.04 mM)	12.0% decrease (1.42 to 1.25 mM)	40.6% increase (32 to 45 mg/L)
			0.0 (OL diet)			
Zock and Katan (34); Mensink *et al.* (33)	30 Women 26 Men Normocholesterolemic	21	7.7	8.5% increase (2.83 to 3.07 mM)	6.8% decrease (1.47 to 1.37 mM)	23.2% increase (69 to 85 mg/L)
			0.1 (LA diet)			
Nestel *et al.* (35)	27 Mildly hypercholesterolemic men	21	6.7	9.5% increased (3.90 to 4.27 mM)	NS change	NS change
			2.4 (OL diet)			
Judd *et al.* (37); Clevidence *et al.* (40)	29 Women 29 Men Normocholesterolemic	42	3.8 (moderate)	6.0% increase (3.34 to 3.54 mM)	NS change	NS change
			6.6 (high)	7.8% increase (3.34 to 3.60 mM)	2.8% decrease (1.42 to 1.38 mM)	NS change
			0.7 (OL diet)			
Judd *et al.* (39)	50 Men Normocholesterolemic	35	4.2 (moderate; TFA/STE)	12.5% increase (2.95 to 3.32 mM)	5.6% decrease (1.24 to 1.17 mM)	NR
			8.3 (high)	13.9% increase (2.95 to 3.36 mM)	6.5% decrease (1.24 to 1.16 mM)	
			0.1 (OL diet)			
Lichtenstein *et al.* (36)	8 Women 6 Men Moderately hypercholesterolemic	32	4.2 (margarine)	8.0% increase (3.23 to 3.49 mM), significant at $P < 0.058$	NS change	NS change
			0.4 (CO diet)			
Lichenstein *et al.* (31)	18 Women 18 Men Moderately hypercholesterolemic	35	0.9 (semiliq. margarine)	NS change	NS change	NS change
			1.3 (butter)	15.1% increase (3.98 to 4.58 mM)	NS change	NS change
			3.3 (soft margarine)	NS change	NS change	NS change
			4.2 (shortening)	6.5% increase (3.98 to 4.24 mM)	NS change	NS change
			6.7 (stick margarine)	9.0% increase (3.98 to 4.34 mM	NS change	NS change
			0.6 (SBO diet)			
Aro *et al.* (41)	49 Women 31 Men Normocholesterolemic	35	8.7	8.3% increase (2.89 to 3.13 mM)	14.1% decrease (1.42 to 1.22 mM)	14.1% increase (270 to 308 mg/L)
			0.4 (STE diet)			
Sundram *et al.* (42)	9 Women 18 Men Normocholesterolemic	28	6.9	20.2% increase (3.17 to 3.81 mM)	16.0% decrease (1.25 to 1.05 mM)	19.5% increase (12.8 to 15.3 mg/dL)
			Not detected (OL diet)			
Wood *et al.* (43)	29 Men Normocholesterolemic	42	6.3 (hard margarine) NR for foods common to all diets	NS change	NS change	NR

(continued)

TABLE 2 (continued)

Reference	Study population	Period (d)	TFA (% en) Treatment and control diets	Change in LDL-C vs. control[a]	Change in HDL-C vs control[a]	Change in Lp(a) vs. control[a]
Almendingen *et al.* (45)	31 Men Normocholesterolemic	19–21	8.5 (PHSBO margarine)	6.0% decrease (3.81 to 3.58 mM vs. butter diet)	NS change vs butter diet	22.7% increase (194 to 238 mg/L vs. butter diet)
			8.0 (PHFO margarine)	NS change vs. butter diet	6.7% decrease (1.05 to 0.98 mM vs. butter diet)	20.6% increase (194 to 234 mg/L vs. butter diets)
			0.9 (butter)			
					Difference between PHSBO (1.05 mM) and PHFO (0.98 mM) diets significant at $P = 0.03$	NS difference between PHSBO and PHFO diets)
Wood *et al.* (44)	38 Men Normocholesterolemic, free living (total diet not controlled)	42	6.7 Minimum (hard margarine)	8.2% decrease (3.78 to 3.47 mM, butter vs. hard margarine diet)	NS change, butter vs. hard margarine diet	NR
			1.2 Minimum (butter)	6.1% decrease (3.47 to 3.26 mM, hard margarine vs. soft margarine diet)	NS change, butter vs. soft margarine diet)	
			0 Minimum (soft margarine)	13.8% decrease (3.78 to 3.26 mM, butter vs. soft margarine diet)	NS difference between hard and soft margarine diets	
Noakes and Clifton (46)	17 Women 21 Men Mildly hypercholesterolemic, free living (total diet not controlled)	21	2.1 min, canola + TFA	12.1% decrease (4.14 to 3.64 mM, butter vs canola + TFA group)	NS change, butter canola + TFA group	NR
			0 min, canola + TFA-free	12.8% decrease (4.14 to 3.61 mM, butter vs. TFA free canola group)	NS change, butter vs. TFA-free canola group	
			0.7 min, butter			
			2.1 min, PUFA + TFA	10.0% decrease (4.70 to 4.23 mM, butter vs. PUFA + TFA group	NS change, butter vs. PUFA + TFA group)	
			0 min, PUFA + TFA-free	15.3% decrease 4.70 to 3.98 mM (butter vs. TFA-free PUFA group)	NS change, butter vs. TFA-free PUFA group	
			0.7 min, butter			
Judd *et al.* (38)	23 Men 23 Women Normocholesterolemic	35	3.9 TFA margarine	4.9% decrease (3.44 to 3.27 mM, butter vs. TFA margarine group)	NS change vs. butter group	8.6% increase (186 to 202 mg/L, butter vs. TFA margarine group)
			2.4 PUFA margarine	6.7% decrease (3.44 to 3.21 mM, butter vs. PUFA margarine group)	NS change vs. butter group	5.9% increase (186 to 197 mg/dL, butter vs PUFA margarine group)
			2.7 butter	Significant difference, 3.27 vs. 3.21 mM, TFA vs. PUFA margarine groups, $P \leq 0.05$	NS difference between TFA and PUFA margarine groups	NS difference between TFA and PUFA margarine groups
de Roos *et al.* (47)	10 Men 19 Women Normocholesterolemic	28	9.2	NS difference after *trans* vs. after saturated diet	21% decrease (1.87 to 1.48 mM after trans vs. after saturated diet	NR
			0.3 (sat diet)			
de Roos *et al.* (48)	11 Men 21 Women Normocholesterolemic	28	9.3	NS difference after *trans* vs. after saturated diet	23% decrease (1.89 to 1.46 mM after *trans* vs. after saturated diet	NR
			0.3 (sat diet)			

(continued)

TABLE 2 (continued)

Reference	Study population	Period (d)	TFA (% en) Treatment and control diets	Change in LDL-C vs. control[a]	Change in HDL-C vs control[a]	Change in Lp(a) vs. control[a]
Denke *et al.* (49)	46 Families: 92 adults, 134 children Normocholesterolemic	35	1.5[b] margarine 0.5 butter	Adults: 11.5% decrease (3.39 to 3.00 mM, after margarine diet vs. after butter diet) Children: 10.8% decrease (2.69 to 2.40 mM, after margarine diet vs. after butter diet)	Adults: NS difference, butter vs. margarine diet Children: NS difference, butter vs. margarine diet	NR
Louheranta *et al.* (50)	14 Women Normocholesterolemic	28	5.1 margarine 0 (OL diet)	NS change	NS change	NS change
Muller et al, (52)	16 Women Normocholesterolemic	14	7.7 (PHFO) 1.1 (veg oil)	9.1% increase (2.63 to 2.87 mM)	NS change	NS change
Cuchel *et al.* (53)	14 Men and women Moderately hypercholesterolemic	32	4.2 (CO stick margarine) 0.4 (CO)	8.0% increase (3.24 to 3.50 mM, $P = 0.058$)	NS change	NR
Han *et al.* (54)	19 Men and women Moderately hypercholesterolemic	32	6.7 (SBO stick margarine) 0.6 (SBO)	11.3% increase (3.88 to 4.32 mM)	6.0 % decrease (1.16 to 1.09 mM)	NR
French *et al.* (55)	10 Men and women Normocholesterolemic	30	5.6 0 (SFA)	11.5% increase (3.23 to 3.60 mM, after TFA vs. after SFA diet)	NS difference between TFA and SFA groups	NR
Lovejoy *et al.* (56)	25 Men and women Normocholesterolemic	28	7.3 0 (OL)	NS change	NS change	NR
Sanders *et al.* (57)	29 Men Normocholesterolemic	14	9.6 0.1 (OL)	NS change	6.3% decrease (1.26 to 1.18 mM)	NS change
Muller *et al.* (51)	27 Women Normocholesterolemic	17	7.0 (PHSBO margarine) 0.2 (PUFA margarine)	13.1% increase (2.61 to 2.88 mM, after PHSBO margarine vs. after PUFA margarine diet)	7.7% decrease (1.43 to 1.32 mM, after PHSBO margarine vs. after PUFA margarine diet)	NS change
Vidgren *et al.* (60)	14 Women Normocholesterolemic	28	5.1 0 (OL)	NR	NR	NR
Sundram *et al.* (61)	10 Women Normocholesterolemic	30	5.6 0 (SFA)	11.5% increase (3.23 to 3.60 mM, after TFA vs. after SFA diet)	7.7% decrease (1.55 to 1.43 mM, after TFA vs. after SFA diet)	NR
Tholstrup *et al.* (59)	42 Men Normocholesterolemic	35	2.1 (vaccenic acid-rich butter) 0.4 (low vaccenic acid butter)	NS change	9.7% decrease (1.54 to 1.39 mM after vaccenic acid-rich diet)	NR

[a]Changes indicated were significant at $P \leq 0.05$ unless otherwise indicated. Abbreviations: TFA, *trans* FA; % en, % of energy; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; Lp(a), lipoprotein(a); OL, oleic acid; LA, linoleic acid; STE, stearic acid; SAT, saturated FA; CO, corn oil; SBO, soybean oil; SO sunflower oil; PHSBO, partially hydrogenated soybean oil; PHFO, partially hydrogenated fish oil; min, minimum (i.e., values apply only to test fat, not to total fat in diet; NS, not significant; NR, not reported.
[b]Based on 3-d food records.

particles. Campos *et al*. (64) previously had reported that large LDL particles were independently associated with increased risk of CHD in case control and prospective studies, after adjustment for established lipid and nonlipid risk factors. In an *in vitro* study, Mitmesser and Carr (65) found that the size of the apoB-100-containing lipoproteins (apoB-100 is the principal apolipoprotein of LDL) secreted by HepG2 cells pre-incubated with elaidic acid or CLA was smaller compared with those pre-incubated with oleic acid or linoleic acid, respectively. This *in vitro* study is consistent with the idea that *trans* FA enhance production of small LDL particles. On the other hand, Kim and Campos (63) stated, "It is possible that the atherogenic characteristics (of small LDL particles) *in vivo* may not be worse than the harmful effects of large LDL." Further research is needed to clarify effects of dietary *trans* FA on LDL particle size.

Collective consideration of human trials. Nine human studies reporting increases in LDL- and decreases in HDL-cholesterol levels when diets high in *trans* FA were compared with diets high in either oleic acid or linoleic acid (11,31,34–37, 39,41,42) have been compared collectively by Ascherio *et al*. (22). (There may have been minor differences among the diets besides the intake of *trans* vs. *cis* monounsaturated FA.) Control diets in these studies contained minute levels of *trans* FA (from zero to 0.7% of energy). Ascherio *et al*. graphed the changes in LDL/HDL ratio reported in these studies against the percentage of energy from either *trans* or saturated FA (Fig. 1). Two best-fit regression lines were plotted through the origin, one line representing the percentage of energy from *trans* FA and the other line, the percentage of energy from saturated FA. Six of the nine studies (11,34,35,37,39,42) allowed a comparison between *trans* and saturated FA. Both regression lines had positive slopes, indicating a positive association between intake of either category of FA and CHD risk. However, the slope of the regression line for *trans* FA was larger (by about twofold) than that for saturated FA. This difference in slopes led to the conclusion that *trans* FA have a more adverse effect on CHD risk than saturated FA (22). This conclusion was supported further by the greater effect on LDL/HDL ratio of *trans* FA compared with saturated FA in the six studies that allowed such a comparison.

Another way to look at these studies collectively is to consider the relationship between simply the change in LDL-cholesterol level or the change in HDL-cholesterol level (rather than the change in their ratio) with increasing level of *trans* FA in the diet (66). This is reasonable because changes in the LDL/HDL ratio do not permit determination of whether LDL, or HDL, or both were changing at a particular dietary level of *trans* FA.

Using data from the same nine studies, Figure 2 is a scatter diagram of the change in LDL-cholesterol with dietary level of *trans* FA. The relationship is directionally similar to that seen when the LDL/HDL ratio was plotted (Fig. 1). Importantly, however, considering these nine studies, the change in LDL-cholesterol was not statistically significant unless the dietary level of *trans* FA was around 4% of energy or higher. The change in HDL-cholesterol with increasing level of *trans* FA

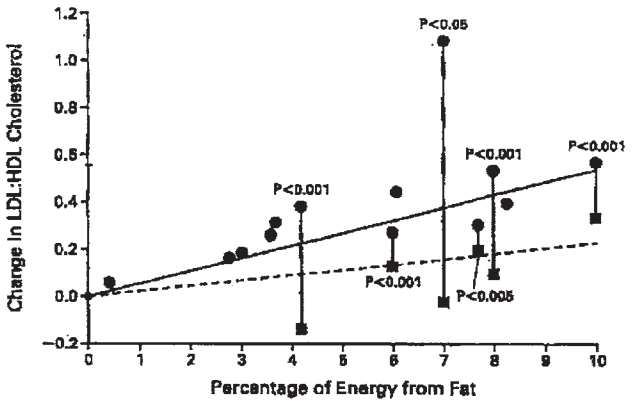


FIG. 1. Change in low density lipoprotein/high density lipoprotein (LDL/HDL) ratio with level of dietary *trans* FA or saturated FA. This graph was reported by Ascherio *et al*. (22) and compares collectively the LDL/HDL ratios against the percentage of energy as either *trans* fat (solid circles, solid line) or saturated fat (solid squares, dashed line) for nine studies (see text). This figure is adapted with permission, copyright© 1999, Massachusetts Medical Society.

in the diet (Fig. 3), with the exception of one study, was not statistically significant unless the dietary level of *trans* FA was higher than about 5–6% of energy. [*Note*: Numerous other human studies (Table 2) that have evaluated effects of *trans* FA on LDL- and HDL-cholesterol levels were excluded from consideration here because they did not compare effects of *trans* FA with those of isocaloric amounts of *cis* FA.]

In an effort to better define the dietary level of *trans* FA that raises LDL-cholesterol and lowers HDL-cholesterol, I considered weighted regression break point analysis and chi-squared analysis (66). Both of these analyses are statistical determinations to estimate possible threshold levels (i.e., "break points"). Weighted regression analysis uses sample size as a weight, thus giving more weight to larger samples. For LDL-cholesterol, a visual break point (the minimum level of *trans* FA resulting in a significant increase in LDL) appeared to exist at or below about 4% of energy as *trans* FA (Fig. 2). Weighted regression analysis, however, did not show quantitatively that 4% of energy is the break point, probably in part because there were only two data points below 3.8% of energy. Nevertheless, it seemed reasonable to expect that a break point might exist in the range of 3.3–4.2% of energy, where the difference between treatment and control LDL values was greatest. This range is consistent with a possible visual break point at or below 4% of energy, i.e., it raises the possibility that there may be a threshold effect of *trans* FA between 3.3 and 4.2% of energy.

In the case of HDL-cholesterol, more data were available below a visual break point estimated to be between 5 and 6% of energy (Fig. 3). However, the absence of data in the range of 4.2–6.6% of energy did not permit more precise estimation of a break point other than to suggest that it might fall in the range of 5–6%.

Chi-squared analyses indicated reasonable break points for effects of *trans* FA on LDL and HDL to be about 3.5 and 7.0% of energy, respectively (66). These latter values are similar to

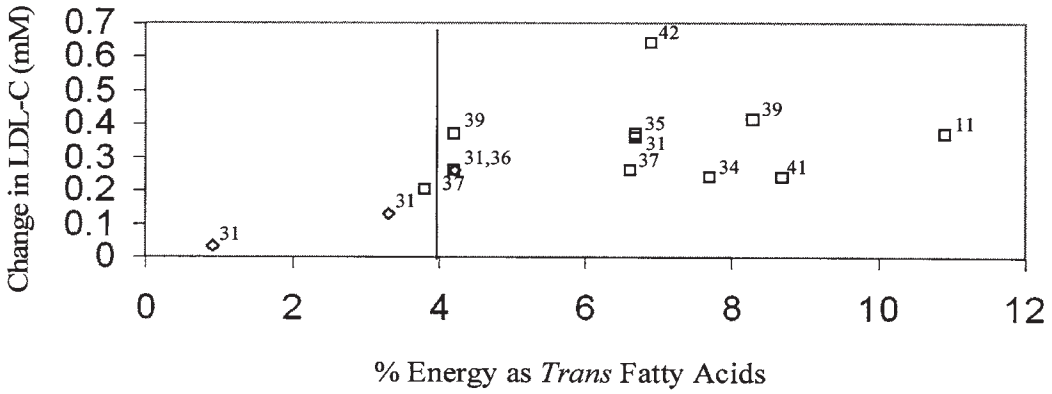


FIG. 2. Scatter diagram of the change in LDL-cholesterol (expressed as mM) with dietary level of *trans* FA (expressed as % energy) using data from the nine studies compared collectively by Ascherio *et al.* (22). Changes in LDL-cholesterol were calculated from the difference between a *trans* FA treatment and its corresponding control treatment. Open diamonds (◇) represent changes in LDL-cholesterol that were reported not to be statistically significant. Open squares (□) represent changes in LDL-cholesterol that were reported to be statistically significant. The number to the right of each point indicates the reference from which the data were taken.

the visual break point estimates of 4% of energy for LDL and 5–6% of energy for HDL.

It is recognized that the confidence in these visual and estimated break points is limited because of the small amount of data (14 treatments) available from the nine studies considered. In particular from Figure 2, there were only two dietary treatments below 4% of energy as *trans* FA. This is an insufficient amount of evidence at present to say whether or not *trans* FA at less than 4% of energy are harmful to humans, and possible biological effects below 4% of energy cannot be ruled out. On the other hand, showing this experimentally would be difficult and expensive and probably not feasible because of the large number of subjects (hundreds or thousands) required along with the difficulty of excluding effects of other FA (saturated, monounsaturated, and polyunsaturated) in the diet. In the case of HDL-cholesterol, lack of data also does not rule out possible biological effects below the apparent visual break point range of 5–6% of energy.

Also because of the limited amount of data available, it is not possible to determine with certainty whether the relation-

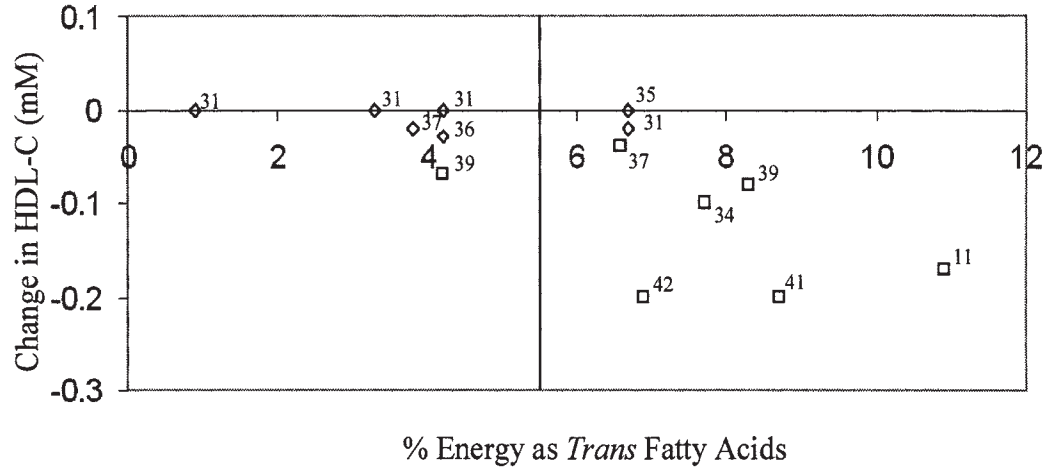


FIG. 3. Scatter diagram of the change in HDL-cholesterol (expressed as mM) with dietary level of *trans* FA (expressed as % energy) using data from the nine studies compared collectively by Ascherio *et al.* (22). Changes in HDL-cholesterol were calculated from the difference between a *trans* FA treatment and its corresponding control treatment. Open diamonds (◇) represent changes in HDL-cholesterol that were reported not to be statistically significant. Open squares (□) represent changes in HDL-cholesterol that were reported to be statistically significant. The number to the right of each point indicates the reference from which the data were taken.

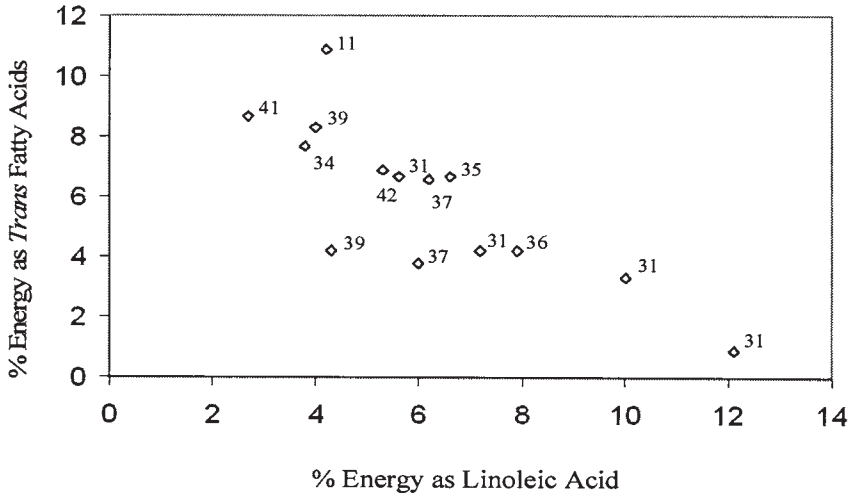


FIG. 4. Scatter diagram comparing the levels of *trans* FA and of linoleic acid used in the *trans* FA diet treatments of the nine studies compared collectively by Ascherio *et al.* (22). Each open diamond (◇) represents the level of dietary *trans* FA and the corresponding level of linoleic acid used in that same treatment. The number to the right of each point indicates the reference from which the data were taken.

ship between the change in LDL-cholesterol (or HDL-cholesterol) and dietary level of *trans* FA is linear, nonlinear, or includes a threshold below which there is no change in LDL-cholesterol with *trans* FA level. Ascherio *et al*. (22) assumed the relationship (considering the LDL/HDL ratio) is linear. Figure 2, however, indicates that the change in LDL-cholesterol may be greatest between 3.3 and 4.2% of energy as *trans* FA and, except for one data point, may be essentially constant between 4 and 11% of energy. However, what is important is not the shape of the curve but the finding that *trans* FA at or above 4% of energy consistently have resulted in significant increases in LDL-cholesterol compared with an essentially *trans*-free diet.

An additional way to consider the same studies is to compare the levels of *trans* FA and of linoleic acid used in the *trans* FA diet treatments (66). A scatter diagram (Fig. 4) indicates that higher dietary levels of *trans* FA were associated with lower levels of linoleic acid and vice versa. In some cases where high levels of *trans* FA (i.e., more than 6% of energy) were fed, the corresponding levels of linoleic acid, 2–4% of energy, while adequate to prevent a nutrient deficiency (e.g., scaly skin), may not have been sufficient to prevent the cholesterolemic effect of a high level of *trans* FA. According to the IOM report (29), an "adequate intake" of linoleic acid for men 31 to 50 yr old is 17 g/d. For men in this age range consuming 2800 kcal/d, this "adequate intake" of linoleic acid would be 5.5% of energy, well above the 2–4% of energy used in several studies. For women 31–50 yr old consuming a 2000 kcal/d diet, the reported "adequate intake" of 12 g/d (29) would provide 5.4% of energy, also above the level used in several studies.

Figure 5 plots the change in LDL/HDL ratio from the *trans* FA diet treatments (22) against the percentage of energy as linoleic acid instead of against the percentage of energy as *trans* FA (66). The regression line obtained is opposite in direction compared with that for the percentage of energy as *trans* FA (22). One interpretation of this difference in direction of the regression lines is that at a sufficient level of linoleic acid in the diet (i.e., 5–6% of energy or higher), *trans* FA may be less effective in increasing the LDL/HDL ratio. Alternatively, increasing dietary linoleic acid may lower the LDL/HDL ratio through the well-known effect of lowering LDL-cholesterol.

A possible physiological explanation for Figure 5 is that *trans* FA and linoleic acid might have opposite effects on the catabolism of the apoproteins of LDL and HDL. Matthan *et al*. (67) reported that dietary hydrogenated fat (i.e., *trans* FA) decreases the catabolism of LDL apoB-100 and increases the catabolism of HDL apoA-I. This could explain the corresponding increase in LDL-cholesterol and decrease in HDL-cholesterol associated with high dietary *trans* FA levels. Perhaps these catabolic effects of *trans* FA are reduced in the presence of sufficient linoleic acid. Additional studies would be needed to clarify whether the proportions of dietary linoleic acid and *trans* FA affect the catabolic rates of the apoproteins.

Note that even at dietary levels of *trans* FA between 4 and 11% of energy, the magnitude of the reported changes in LDL-cholesterol between *trans* and essentially *trans*-free diets was small, ranging from 0.20 to 0.64 mmol/L (equivalent to 7.7 to 25 mg/dL). Similar small changes in LDL-cholesterol might reasonably be expected in many people from the near elimination from the diet of industrially produced *trans* FA. Although small reductions in LDL-cholesterol in a population may benefit heart health, the suggestion by Mozaffarian *et al*. that the near elimination of industrially produced *trans* FA would decrease the number of CHD events each year by up to 228,000 (30) seems to be greatly exaggerated. Achieving such a major reduction in CHD events likely would require additional and more drastic lifestyle changes (e.g., elimination of smoking and greater participation in regular exercise) and more widespread use of cholesterol-lowering drugs.

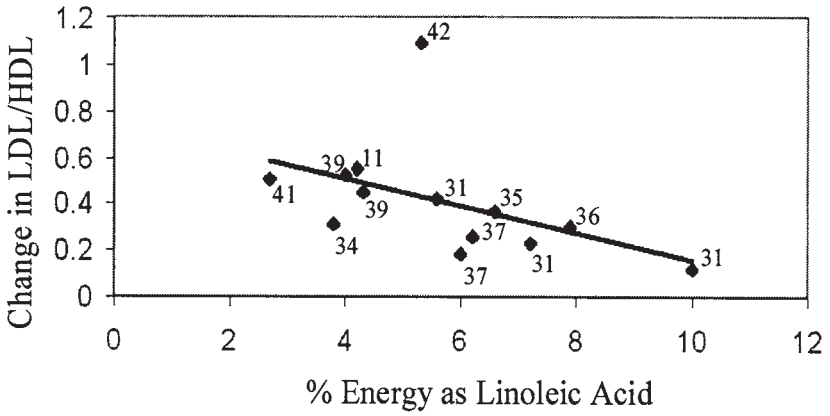


FIG. 5. Change in the ratio of LDL-cholesterol/HDL-cholesterol vs. the dietary level of linoleic acid using data from the nine studies compared collectively by Ascherio *et al.* (22). The slope for the best-fit regression line is –0.059, similar in magnitude (0.056) to that determined for the graph presented by Ascherio *et al.* (22; LDL/HDL ratio vs. % energy as *trans* FA) but opposite in sign. The number to the right of each point indicates the reference from which the data were taken.

In summary, it appears that at a sufficiently high dietary level, *trans* FA consistently raise LDL-cholesterol and reduce HDL-cholesterol levels compared with a diet essentially free of *trans* FA. The dietary levels of *trans* FA necessary to increase LDL-cholesterol appear to be around 4% of energy and higher and to decrease HDL-cholesterol, around 5–6% of energy or higher compared with control diets. Thus, high dietary levels of *trans* FA indeed increase CHD risk. On the other hand, because of limited available data, it was not possible to estimate precise break points for effects of *trans* FA on LDL or HDL. There is insufficient evidence at present to say whether *trans* FA at less than 4% of energy in the diet are or are not harmful to humans.

Studies on clotting tendency, blood pressure, LDL oxidation, flow-mediated vasodilation (FMD), and systemic inflammation. Results of studies assessing the effect of dietary *trans* FA on blood clotting tendency have been inconsistent. Wood *et al.* (43) reported that blood levels of thromboxane B_2, an indicator of increased *in vitro* platelet aggregation (i.e., a measure of increased clotting tendency), were unchanged in a group of human subjects fed high levels of hard margarine compared with subjects fed a baseline diet. Almendingen *et al.* (68) found that a diet containing 8.5% of energy from a margarine made from partially hydrogenated soybean oil resulted in higher levels of plasminogen activator inhibitor type 1 than a diet containing 8.0% of energy from a margarine made from partially hydrogenated fish oil. This finding suggested that partially hydrogenated soybean oil had unfavorable antifibrinolytic effects compared with partially hydrogenated fish oil. There were no differences between the diets in levels of factor VII, fibrinogen peptide A, β-thromboglobulin, D-dimer, or tissue plasminogen activator.

In other studies, Mutanen and Aro (69) reported no differences in coagulation and fibrinolysis between a high stearic acid diet (0.4% of energy as *trans* FA) and a high *trans* FA diet (8.7% of energy as *trans* FA). The same high stearic acid and *trans* FA diets also produced similar effects on platelet aggregation and endothelial prostaglandin I_2 production (70). Sanders *et al.* (71) saw no significant differences in factor VII coagulation activity after feeding single meals high in either oleic acid (0.07% of energy as *trans* FA) or elaidic acid (24.7% of energy as *trans* FA). A subsequent study (57) in which normocholesterolemic men were fed a high oleic acid diet (0.1% of energy as *trans* FA) or a high *trans* FA diet (9.6% of energy as *trans* FA) for 2 wk resulted in no differences in known hemostatic risk markers (fibrinogen and D-dimer concentrations, factor VII coagulant, plasminogen activator inhibitor type 1, and tissue plasminogen activity) for cardiovascular disease. Feeding a diet high in the ruminant *trans* FA vaccenic acid (1.1% of energy) similarly had no effect on the hemostatic variables factor VII coagulant activity and plasminogen activator inhibitor type 1 (59).

With regard to blood pressure, two studies with normotensive subjects showed no effect of *trans* FA (fed at up to 10.9% of energy) compared with oleic acid on either systolic or diastolic blood pressure (72,73). Similarly, high-*trans* diets containing up to 8.5% of energy as *trans* FA consistently have been reported to produce little or no effect on the susceptibility of LDL to oxidation (35,53,74,75).

FMD is defined as the increase in diameter of an artery, usually the brachial artery in the arm, after a provoked local increase in blood flow. Impaired FMD appears to be predictive of future coronary events. De Roos *et al.* (47,76) found that dietary replacement of saturated FA by *trans* FA for 4 wk reduced FMD by 29% and decreased HDL-cholesterol (Table 2). Replacement of monounsaturated fats by carbohydrates did not impair FMD, but it decreased HDL-cholesterol. Acute postprandial impairments of FMD by either *trans* or saturated FA were not found, suggesting that long-term effects are re-

sponsible for reported detrimental effects of *trans* FA on health.

Systemic inflammation is involved with a variety of neurological and degenerative conditions and is considered a nonlipid risk factor for CHD, insulin resistance, diabetes, and heart failure. Chronic systemic inflammation is tissue-destructive, and cardiovascular tissue is no exception. Low-grade systemic inflammation can be measured in blood with the inflammatory marker C-reactive protein (CRP). High levels of CRP have been associated with increased incidence of myocardial infarction and with stroke. Elevated concentrations of interleukin 6 (IL-6) and soluble tumor necrosis factor alpha receptors 1 and 2 (sTNF-R1 and sTNF-R2) have been associated with insulin resistance, diabetes, and heart disease.

Mozaffarian *et al*. (30,77–79) have suggested that dietary *trans* FA may influence systemic inflammation, which might partly account for the relationship between *trans* FA and heart disease. Mozaffarian *et al.* (78) investigated the relationship between *trans* FA intake (measured by food frequency questionnaires) and inflammatory markers in healthy female subjects. *Trans* FA intake was positively associated with sTNF-R1 and sTNF-R2 but not with IL-6 or CRP concentrations overall. *Trans* FA intake was associated with IL-6 and CRP in women with higher body mass index. In patients with established heart disease (79), *trans* FA levels of red blood cell membranes (a measure of dietary intake) were associated with activation of systemic inflammatory responses, including increased levels of IL-6 and TNF receptors. In a controlled feeding trial with 50 men, a high *trans* FA diet (8% of energy) raised plasma CRP levels compared with carbohydrate- or oleic acid-containing diets (80). Further investigation of dietary *trans* FA in relation to inflammation and the implications for coronary disease and other conditions is warranted.

In summary, a limited number of studies have reported no consistent effects of dietary *trans* FA on blood clotting tendency and no significant effects on blood pressure or LDL oxidation compared with dietary *cis* FA. Several studies have suggested a possible relationship between dietary *trans* FA and systemic inflammation, but the potential mechanism of such an effect is uncertain.

Epidemiologic and case-control studies. Several epidemiologic and case-control studies have reported associations between the intake of *trans* FA and the risk of developing CHD (81–89). Key study design aspects and results of these studies are summarized in Table 3. Results of the studies were consistent in that *trans* FA intake (usually assessed by a food frequency questionnaire) was positively associated with risk of CHD or with a major coronary event, particularly when relative risks between the highest and lowest quintiles of intake were considered.

On the other hand, in at least one study (83), individuals eating intermediate levels of *trans* FA had a lower risk of CHD compared with individuals eating the *lowest* level of *trans* FA. This study did not support a dose-response relationship. A limitation of epidemiologic studies is that they may show associations between two variables but they do not prove cause-and-effect relationships. The relatively low risk values reported for associations of *trans* FA intake and CHD risk (typically around 1.2–1.5, Table 3) are considered weak associations by many epidemiologists [Lilienfeld and Stolley (90); Rothman and Greenland (91)].

Dietary *trans* FA have been considered in relation to sudden death from cardiac causes in at least two case-control studies. A study by Roberts *et al*. (92) compared proportions of *trans* isomers of oleic and linoleic acid in adipose tissue obtained at autopsy from 66 subjects with sudden death from cardiac causes with adipose tissue from 286 healthy control subjects. No relationship was seen between combined *trans* isomers of oleic and linoleic acid and sudden cardiac death. When the individual FA were considered, *trans* oleic acid was negatively associated with risk of sudden cardiac death, and no association was seen with *trans* forms of linoleic acid. Overall, this study did not support the hypothesis that *trans* isomers increase the risk of sudden cardiac death. In contrast, a study by Lemaitre *et al*. (93; discussed in 94) reported that the level of total *trans* FA in red blood cell membranes (a biomarker of dietary intake) was associated with a modest increase in risk of primary cardiac arrest. However, when different *trans* isomers were assessed separately, the isomers of oleic acid were not associated with risk, whereas those of linoleic acid were associated with a threefold increase in risk. It seems doubtful, however, that *trans* 18:2 isomers are present at sufficient levels in typical U.S. diets to contribute uniquely and significantly to the risk of primary cardiac arrest (95). More work is needed to establish whether *trans* isomers are associated with an increased risk of sudden cardiac death.

A further limitation of epidemiologic studies is that self-reported dietary intake data are likely inaccurate. Mertz *et al*. (96) found that recall and food record data usually underestimate intake. A further complicating factor is that the *trans* FA contents of similar-appearing foods, such as tub margarines, can be highly variable among brands and even with the same brand over time. Thus, an assumed *trans* FA level for the category of "tub margarines" may not result in accurate calculation of *trans* FA intake levels over time.

Additionally, a recent report by Schatzkin *et al.* (97) does not recommend use of a food frequency questionnaire for evaluating relationships between absolute intake values and disease. This is because repeated applications of the questionnaire did not lead to improvement in proposed relationships being considered. The study by Schatzkin *et al.* (97) was part of the National Cancer Institute's "Observing Protein and Energy Nutrition" (OPEN) Study designed to assess dietary measurement error by comparing results from self-reported dietary intake data with four dietary biomarkers based on analysis of urine: doubly labeled water and urinary nitrogen, sodium, and potassium. A key finding was significant underreporting of energy and protein intakes by both men and women. Although the study by Schatzkin *et al*. (97) did not assess FA intake, this study nevertheless would raise questions about the strength of

TABLE 3
Epidemiological/Case Control Studies Relating Dietary *trans* FA and Risk of Coronary Heart Disease[a]

Reference	Study design	Subjects	TFA intake	Results	Comments
Troisi *et al.* (81)	Cross-sectional	748 men, aged 43–85 yr	Food frequency questionnaire; for entire sample, mean TFA intake was 1.6% of total energy	TFA intake directly related to total serum (r = 0.07, P = 0.04) and LDL-C (r = 0.09, *P* = 0.01).	Authors suggested increasing TFA intake from 2.1 to 4.9 g/d would correspond to a 27% increase in risk of MI.
Willett *et al.* (82)	Prospective cohort study among 85,095 women	431 women experiencing new CHD (nonfatal MI or death from CHD) over 8-yr follow-up period	Food frequency questionnaire; quintiles of mean TFA intakes: 1.3, 1.8, 2.2, 2.6, and 3.2% of energy (2.4, 3.2, 3.9, 4.5, and 5.7 g/d)	TFA intake directly related to risk of CHD (relative risk for highest vs. lowest quintile was 1.50 (p = 0.001 for trend).	Authors suggested consumption of partially hydrogenated vegetable oils may contribute to occurrence of CHD.
Ascherio *et al.* (83)	Case-control	239 MI patients, 282 population controls	Food frequency questionnaire; quintiles of mean TFA intake: 1.69, 2.48, 3.35, 4.52, 6.51 g/d	TFA intake directly related to risk of MI [relative risk for highest vs. lowest quintile was 2.44 (P < 0.0001 for trend)]. Increased risk evident only among individuals in top quintile of intake.	Patients not asked whether they changed their dietary intakes after their MI. LDL-C in this group may reflect recent dietary intakes rather than diet before MI.
Kromhout *et al.* (85)	Cohort study among men from 7 countries	12,763 men, aged 40–59 yr, over 25-yr follow-up period	Food composites collected and analyzed for TFA. Average TFA intakes: 0.05 to 1.84% of energy	Strong positive associations between 25-yr death rates from CHD and average intake of TFA (elaidic acid, r = 0.78, *P* < 0.001).	Authors suggested dietary TFA (also SFA and cholesterol) are important determinants of differences in population rates of CHD death.
Ascherio *et al.* (84)	Cohort study	43,757 men, aged 40–75, over 6-yr follow-up period	Food frequency questionnaire; median intakes (g/d) of quintiles: 1.5, 2.2, 2.7, 3.3, 4.3 (0.8 to 1.6% of energy, lowest to highest quintiles)	TFA intake directly associated with risk of MI (nonfatal and fatal). Relative risk for quintiles: 1.0, 1.20, 1.24, 1.27, 1.40, P = 0.01.	The association was attenuated after adjustment for dietary fiber.
Hu *et al.* (86)	Prospective cohort study	939 women with MI, aged 34–59 yr	Food frequency questionnaire; median intakes of quintiles (% of energy): 1.3, 1.7, 2.0, 2.4, 2.9	TFA intake directly associated with risk of CHD. Relative risk for quintiles: 1.0, 1.07, 1.10, 1.13, 1.27 (P= 0.02 for trend).	Authors reported that replacing 2% of energy intake from TFA with unhydrogenated, unsaturated fats would reduce CHD risk by 53%.
Pietinen *et al.* (87)	Cohort study	21,390 male smokers, aged 50–69 yr, over 6.1-yr follow-up period	Food frequency questionnaire; median intakes (g/d) of quintiles: 1.3, 1.7, 2.0, 2.7, 6.2	TFA intake significantly associated with risk of major coronary event. Relative risk for quintiles: 1.00, 1.10, 0.97, 1.07, 1.14 (P = 0.158 for trend).	Authors also reported positive association between TFA intake and risk of coronary death (*P* for trend = 0.004).
Tavani *et al.* (88)	Case-control	Italian women, aged 18–74 yr, 429 MI cases, 866 controls	Margarine intakes reported as no or low, medium, or high.	Medium or high intake of margarine associated with increased risk of MI [multivariate odds ratio (OR) = 1.5 vs. no or low intake. Association stronger for women aged 60–74 yr (OR = 2.4) and in current smokers (OR = 2.3).	Authors suggested the association with margarine could explain about 6% of MI in this population. Major limitation of this study: low intake of margarine by these Italian women.
Oomen *et al.* (89)	Prospective study	667 elderly Dutch men, aged 64–84 yr	Dietary surveys; average TFA intakes fell from 10.9 to 6.9 to 4.4 g/d from 1985 to 1990 to 1995 (4.3, 2.9, and 1.9% of energy)	TFA intake positively associated with 10-yr risk of CHD. Relative risk for a difference of 2% of energy in TFA intake at baseline was 1.28.	Authors suggested that high intake of TFA contributes to risk of CHD.
Lemaitre *et al.* (93)	Case-control	174 cardiac arrest patients, 285 controls, aged 25–74 yr	TFA levels in red blood cell membranes used as indicator of intake.	Higher TFA levels in red blood cell membranes associated with increased risk of primary cardiac arrest (relative risk = 1.47). *Trans* isomers of oleic acid not associated with risk, but those of linoleic acid associated with threefold increase in risk.	It seems unlikely that *trans* 18:2 isomers are present at sufficient levels in typical U.S. diets to contribute significantly to risk of primary cardiac arrest.

[a]Abbreviations: CHD, coronary heart disease; MI, myocardial infarction; TFA, *trans* FA; SFA, saturated FA; LDL-C, LDL-cholesterol; *r* = partial correlation coefficient (adjusted for various parameters including energy intake, age, body mass index, waist-to-hip ratio, smoking status, and alcohol intake).

the proposed relationship between CHD risk and *trans* FA intake because most studies reporting such a relationship used a food frequency questionnaire to assess *trans* FA intake. (Further information about the OPEN Study may be found at the NCI website: http://riskfactor.cancer.gov/studies/open/status.html)

Correlation coefficients between FA intake data estimated from food frequency questionnaires and plasma FA generally have been higher for PUFA as well as n-3 FA of marine origin than for saturated and monounsaturated FA. In a large population of free-living, middle-aged adults, Ma *et al*. (98) found correlation coefficients between dietary and plasma FA (expressed as the percentage of total FA) for phospholipid and cholesterol ester FA, respectively, to be as follows: total PUFA (r = 0.25 and 0.31), linoleic acid (r = 0.22 and 0.28), α-linolenic acid (r = 0.15 and 0.21), eicosapentaenoic acid (EPA, r = 0.20 and 0.23), docosahexaenoic acid (DHA, r =0.42 and 0.42), saturated FA (r = 0.15 and 0.23), and monounsaturated FA (r = 0.05 and 0.01). The correlations between diet and plasma FA held relatively constant regardless of whether subjects were overweight, had chronic diseases, were alcohol drinkers, or were cigarette smokers. In a population of Norwegian men, Andersen *et al*. (99) reported correlation coefficients between dietary FA and FA in total serum lipids to be: linoleic acid, 0.16; α-linolenic acid, 0.28; EPA, 0.51; DHA, 0.52; palmitic acid, 0.11; and monounsaturated FA, 0.08. In a group of British civil servants (men and women), Brunner *et al*. (100) reported correlations between dietary intakes and FA in serum cholesteryl esters to be: EPA, 0.54; linoleic acid, 0.38; total PUFA, 0.43; and total saturated FA, –0.01. Because plasma monounsaturated and saturated FA may arise from endogenous monounsaturated and saturated FA, as well as from the diet, food frequency questionnaires may not always provide reliable estimates of dietary monounsaturated and saturated FA.

Other studies. Muller *et al*. (101) and Pedersen *et al*. (102) developed regression equations to predict effects on serum total and LDL-cholesterol levels of dietary *trans* FA as well as individual saturated FA. The regression equations were based on consideration of four controlled studies. The food sources of *trans* FA were partially hydrogenated soybean oil and partially hydrogenated fish oil. *Trans* FA from these two sources differ with respect to chain length and number of *trans* bonds. The authors concluded that myristic acid (14:0) is the most hypercholesterolemic FA and that *trans* FA are less hypercholesterolemic than the saturated FA myristic and palmitic (16:0) acids. Hydrogenated fish oil was felt to be slightly more hypercholesterolemic than hydrogenated soybean oil. Separately, Lichtenstein *et al*. (31) reported predictive equations for total and LDL-cholesterol and also for HDL-cholesterol.

Kritchevsky (103) cited human data indicating that if the diet contains a high level of linoleic acid (e.g., 22–37% of total dietary FA) and a relatively low level of *trans* FA (e.g., 8–18% of total dietary FA), then the change in total serum cholesterol is much smaller than if the diet contains a low level of linoleic acid and a high level of *trans* FA. Such a relationship would be predicted according to equations presented by Muller *et al.* (101). The relationship is supported by data cited in Figures 4 and 5 (see also Ref. 66).

The *trans* fats used in studies considered thus far have contained principally *trans* 18:1 isomers as well as low levels of *trans* 18:2 isomers. These fats have been essentially free of *trans* 18:3 isomers. Vermunt *et al*. (104) recently investigated the effect of *trans* α-linolenic acid formed by deodorization of canola oil (105) on plasma lipids and lipoproteins. Eighty-five healthy men from three European countries consumed α *trans* FA-free diet for 6 wk followed by either a diet "high" or "low" in *trans* α-linolenic acid for 6 wk. Daily mean total *trans* α-linolenic acid intake was 1410 mg in the high-*trans* group and 60 mg in the low-*trans* group. The high *trans* α-linolenic acid diet did not change total, LDL-, or HDL-cholesterol levels, but it did result in statistically significant increases in the plasma LDL/HDL ratio and the total/HDL ratio compared with the low *trans* diet. The authors commented that whether the diet-induced changes in these ratios truly affect CHD risk remains to be established. These results, however, suggest the importance of selecting conditions for deodorization that minimize formation of *trans* α-linolenic acid isomers. A parallel study by Armstrong *et al*. (106) using the same high dietary level of *trans* α-linolenic acid found no effect on platelet aggregation and blood coagulation factors, including platelet thromboxane production, fibrinogen levels, factor VII, activated factor VIIa, or plasminogen activator inhibitor activity.

The level of *trans* α-linolenic acid produced by deodorization of canola or soybean oil depends on the time and temperature of the deodorization process (107). The canola oil used in the studies by Vermunt *et al.* (104) and Armstrong *et al*. (106) contained 4.5% *trans* α-linolenic acid and was produced by batch deodorization for 52.5 h at 205°C (105). In contrast, most canola oil produced in the United States has a *trans* α-linolenic acid level ranging from about 1.0 to 2.5% (personal communication, David Volker, J.M. Smucker Co., Cincinnati, OH). The lower (1.0%) level of *trans* α-linolenic acid typically is produced by a continuous deodorization process lasting up to about 5 min at about 240–270°C. The higher (2.5%) level typically is produced by batch deodorization for up to 2 h also at 240–270°C. Canola oil containing 2.5% *trans* α-linolenic acid would qualify for a label claim of zero *trans* FA in the United States, because a 14-g (1 tablespoon) serving of the oil would provide 0.35 g of *trans* FA. The U.S. FDA permits a label claim of zero *trans* FA if the food product contains less than 0.5 g of *trans* FA per serving.

The question has been raised as to whether *trans* FA from ruminant fats have similar metabolic effects to those from industrially hydrogenated fats. Controlled metabolic trials have not directly addressed this question. The predominant *trans* isomer of ruminant fats is vaccenic acid, an 18-carbon monounsaturated FA with its double bond at the 11-position of the carbon chain (18, *t*-11). In contrast, the *trans* isomers of industrially hydrogenated fats have their double bonds primarily at positions 9, 10, and 11 (108). Meijer *et al*. (109) reported that feeding vaccenic acid (in mixed TG) to hamsters resulted in a signifi-

cant increase in the LDL-/HDL-cholesterol ratio compared with feeding elaidic acid (18, *t*-9). This study suggested that elaidic acid might be preferable to vaccenic acid with respect to reducing CHD risk. On the other hand, an overview of effects of ruminant *trans* FA by Jakobsen *et al*. (110) noted that some epidemiological studies have suggested an innocuous or even protective effect of ruminant *trans* FA (81,86) whereas other such studies have not (88). Jakobsen *et al*. (110) concluded that controlled metabolic studies on effects of intake of total and specific ruminant *trans* FA on CHD risk factors are warranted. In addition, they suggested that epidemiological studies on the intake of ruminant *trans* FA and risk of CHD in populations with high intake of ruminant *trans* FA also are warranted.

In summary, since 1990, controlled human studies have found that sufficiently high levels of dietary *trans* FA increase LDL-cholesterol and decrease HDL-cholesterol compared with diets high in *cis* monounsaturated FA or PUFA. The dietary levels of *trans* FA necessary to detect this effect appear to be approximately 4% of energy or higher to increase LDL-cholesterol and approximately 5–6% of energy or higher to decrease HDL-cholesterol, compared with control diets that are essentially *trans*-free. There are very limited data at lower levels of intake (less than 4% of energy). In the United States, the intake of saturated FA is reported to be substantially higher (about 10–12% of energy) than the intake of *trans* FA (about 2–3% of energy). Also since 1992, several epidemiologic and case-control studies have reported a positive association between dietary *trans* FA and the risk of CHD. A major limitation of the epidemiologic studies may be inaccurate *trans* FA intake data. To date, no consistent effects of dietary *trans* FA compared with *cis* FA have been reported on blood clotting tendency, blood pressure, LDL oxidation, or sudden death, and it is not clear whether ruminant *trans* FA have different metabolic effects compared with industrially produced *trans* FA.

STUDIES RELATING DIETARY *TRANS* FA TO CANCER, MATERNAL AND CHILD HEALTH, TYPE 2 DIABETES, AND MACULAR DEGENERATION

Trans FA in relation to cancer. In contrast to the extensive literature on *trans* FA in relation to CHD, relatively few investigators have studied *trans* FA with respect to cancer. Ip and Marshall (111) reviewed comprehensively more than 30 reports addressing the strength and consistency of available scientific data on *trans* FA and cancer. One reviewed study (112) involved the treatment of a colon cancer-sensitive strain of rats with the colon carcinogen dimethylhydrazine and then compared tumor development after feeding diets containing either partially hydrogenated corn oil or high-oleic safflower oil. Overall, there was no evidence that cancer of the intestinal tract was differentially affected by fat type given to animals of either sex.

Ip and Marshall (111) also evaluated epidemiologic studies since around 1990 that have considered the influence of the intake of various types of FA on the development of cancer at different sites. For breast cancer, Ip and Marshall noted that the epidemiologic evidence shows only a slight to negligible effect of fat intake in general on breast cancer risk and no strong evidence that the intake of *trans* FA is related to increased risk. For colon cancer, evidence exists of increasing risk with the intake of saturated fat or animal fat and decreasing risk with increased intake of fiber and vegetable products. However, no evidence indicated that the intake of *trans* FA is related to an increased risk of colon cancer or rectal cancer. For prostate cancer, Ip and Marshall noted evidence that fat intake might be related to risk. Most of this evidence has focused on the consumption of saturated and animal fats. The intake of *trans* FA has not been correlated with prostate cancer risk.

Since the review by Ip and Marshall (111), two case-control studies have investigated an association between dietary *trans* FA and the development of cancer. Kohlmeier *et al*. (113) investigated the relationship between adipose tissue levels of *trans* FA and postmenopausal breast cancer in European populations in 209 cases and 407 controls. Adipose tissue levels of *trans* FA were used as a biomarker of dietary exposure. The adipose concentration of *trans* FA showed a positive association with breast cancer, suggesting a relationship between adipose stores of *trans* FA and postmenopausal breast cancer risk in European women. Dietary FA data, however, were not provided to relate to adipose tissue stores.

The other case-control study was conducted by Slattery *et al*. (114) to examine a possible association between dietary *trans* FA and colon cancer incidence in 1,993 cases and 2,410 control subjects. Dietary data were collected using a diet history questionnaire. The authors found a weak association between dietary *trans* FA and colon cancer risk in women (odds ratio = 1.5) but not in men (odds ratio = 1.2). Slightly stronger associations were observed in subjects aged 67 yr or older (odds ratios = 1.4 for men, 1.6 for women). The authors concluded that it seems prudent to avoid consuming partially hydrogenated fats, because no increased risk was observed for *cis* FA.

In recent years, a specific category of *trans* FA, conjugated linoleic acid (CLA, a group of isomers of linoleic acid, each with one *trans* bond), has been reported to have possible human health benefits, including controlling body fat gain, enhancing immunity, and reducing inflammation. Recent perspectives on CLA have been discussed by Pariza (115; see additional related articles in this *Am. J. Clin. Nutr.* supplement). CLA is not further discussed in this article so that the focus here can be on effects of *trans* FA produced as a result of partial hydrogenation of oils.

In summary, current scientific evidence does not support a convincing relationship between *trans* FA intake and the risk of cancer at specific sites. Two recent case-control studies have reported an association between the intake of *trans* FA and breast cancer or colon cancer risk. However, the accuracy of the intake values is subject to question.

Trans FA in relation to maternal and child health. Concerns about a possible relationship between dietary *trans* FA and maternal and child health have focused on whether *trans* FA may affect human fetal growth and infant development. It is well established that n-6 and n-3 long-chain PUFA play significant roles in growth and development. Linoleic acid (18:2n-6) and

α-linolenic acid (18:3 n-3) are EFA (i.e., they cannot be synthesized by humans) are precursors of other important long-chain PUFA. Specifically, arachidonic acid (20:4n-6) is derived from linoleic acid by desaturation and chain elongation reactions and is a component of membrane phospholipids, a precursor for eicosanoids, and a stimulant of growth and cell division. DHA (22:6 n-3) is obtained in the diet (e.g., from fish oils) or synthesized from α-linolenic acid (also by desaturation and chain elongation) and is a component of retinal and neural membrane phospholipids. As such, it is involved in visual and central nervous system function.

According to Carlson *et al*. (116), concerns have been expressed that *trans* FA could decrease fetal and neonatal tissue concentrations of n-6 and n-3 long-chain PUFA, thereby interfering with normal growth and development. Carlson *et al*. (116) concluded that a definitive answer to the question of whether *trans* FA affect fetal development is not possible because there are few studies in this area, observed effects have been small, results have been inconsistent, and confounding factors have not been excluded.

Noting that *trans* FA from the maternal diet can cross the placenta or be secreted into human milk, Craig-Schmidt (117) indicated that developing fetuses and nursing infants in the United States and Canada generally would be exposed to larger amounts of *trans* FA than would infants in other parts of the world. This is supported by larger amounts of *trans* isomers in human milk samples from the United States and Canada compared with human milk samples from various countries of Europe, Asia, or Africa.

Craig-Schmidt (117) also cited studies in which plasma *trans* FA levels were inversely related to birth weight and head circumference. She added that the hypothesis that dietary *trans* FA could inhibit the biosynthesis of long-chain PUFA with 20 or 22 carbon atoms and thus affect infant development is supported by studies demonstrating an inverse correlation between plasma *trans* FA and n-3 and n-6 long-chain PUFA in infants. However, no such relationship has been observed in human milk. Craig-Schmidt (117) concluded that the evidence supporting an unfavorable effect of *trans* FA on infant development is correlational and that the physiological implications of the reported relationships are largely unknown.

Larque *et al*. (118) also reviewed studies on the effects of dietary *trans* FA in early life. These authors added that multigenerational studies using animals have shown no correlation between birth weight or growth and dietary *trans* FA. Citing reports of inverse relationships between plasma *trans* FA, arachidonic acid, and DHA, Larque *et al*. (118) suggested that such results may indicate an inhibitory effect of *trans* FA on liver Δ6 FA desaturase activity. In contrast to blood and liver, the brain appears to be protected from *trans* FA accumulation in experimental animals, but no data have been reported for human infants.

Decsi *et al*. (119) evaluated previously reported inverse correlations between values of *trans* FA and long-chain PUFA in plasma lipids of full-term infants. In this study, the FA composition of venous cord blood lipids was determined in 42 healthy full-term infants. The investigators found significant inverse correlations between total *trans* FA and both arachidonic acid and DHA in phospholipids, cholesterol esters, and nonesterified FA, but not in TG. Because *trans* FA in the fetal circulation originate from the maternal diet, the authors suggested that maternal exposure to *trans* FA may inversely influence long-chain PUFA status in full-term infants at birth.

The Institute of Medicine report (29) recognized concerns that *trans* FA have been suggested to have adverse effects on growth and development through inhibition of the desaturation and elongation of linoleic and α-linolenic acids to arachidonic acid and DHA, respectively. However, considering available evidence, including experimental work showing that inhibition of the Δ6 desaturation of linoleic acid is not of concern with linoleic acid intakes above about 2% of energy, the IOM concluded that inhibition of EFA metabolism by *trans* FA is unlikely to be of concern for practical human diets.

A recent study by Friesen and Innis (120) indicated that the level of total *trans* FA in human milk samples in Canada decreased significantly from 7.1 g/100 g FA in 1998 to 4.6 g/100 g FA in early 2006. These results suggested a concomitant decrease in *trans* FA intake among lactating women and breastfed infants. This decrease in intake could be related in part to the introduction of *trans* fat labeling of retail foods in Canada in 2003 and also in part to recent efforts by edible oil manufacturers to reduce or eliminate *trans* FA from their products (to be discussed later in this paper). Innis (121) also has recommended the removal of *trans* FA from industrial sources from the diets of pregnant and lactating women and young children, while improving dietary intakes of n-3 FA.

In summary, it appears unlikely that current average dietary levels of *trans* FA in the United States and Europe will have unfavorable effects on growth and development, reproduction, or gross aspects of fetal development. Future studies should address the possible benefits to human growth, development, and health of current efforts by the food industry to reduce or eliminate *trans* FA from their products.

Trans FA in relation to type 2 diabetes. Salmeron *et al*. (122) suggested that an increase in PUFA intake and a decrease in *trans* FA intake would substantially reduce the risk of women developing type 2 diabetes. During a 14-yr follow-up of 84,204 women with no diabetes, cardiovascular disease, or cancer, the investigators documented 2,507 new cases of type 2 diabetes. Dietary information was assessed by food frequency questionnaires. Total fat intake, compared with equivalent energy intake from carbohydrates, was not associated with risk of type 2 diabetes. In contrast, PUFA intake was inversely associated and *trans* FA intake positively associated with risk. The authors estimated that replacing 2% of the energy from *trans* FA with PUFA would reduce the risk of type 2 diabetes by 40%.

Commenting on this study, Clandinin and Wilke (123) noted that the influence of dietary *trans* FA on insulin resistance has not been studied, and epidemiologic evidence relating *trans* FA intake to diabetes is lacking. There is no known functional or physiological relation to connect *trans* FA to disease mechanisms involved with type 2 diabetes. Clandinin and Wilke also

questioned the reliability of estimates of intake of *trans* FA from food frequency questionnaires.

An additional complication is that some processed foods containing *trans* FA also contain large amounts of refined carbohydrates (e.g., certain baked goods), which could exacerbate the insulin-resistant state in diabetes and/or contribute to increases in serum TG levels. This association of *trans* FA with simple and complex carbohydrates does not permit distinction about whether *trans* FA alone, *trans* FA plus carbohydrates, or carbohydrates alone in certain products would contribute to the risk of type 2 diabetes. Thus, the conclusions of Salmeron *et al*. (122) still represent a hypothesis to be tested.

A follow-up study by Hu *et al*. (124) looked at combined effects of dietary and lifestyle factors in relation to type 2 diabetes. Considering essentially the same subject population studied by Salmeron *et al*. (122), Hu *et al*. (124) documented 3,300 new cases of type 2 diabetes during 16 yr of follow-up. Overweight or obesity was the single most important predictor of diabetes. Lack of exercise, a poor diet, current smoking, and abstinence from alcohol use were all associated with increased risk of diabetes, even after adjusting for body mass index. Also, the combination of an appropriate diet, moderate amount of exercise, and abstinence from smoking, even in overweight and obese subjects, resulted in reduced risk of type 2 diabetes. A "poor diet" was described as being high in *trans* fat and glycemic load, low in cereal fiber, and having a low ratio of polyunsaturated to saturated fat. Quantitative levels of *trans* FA, sugars, cereal fiber, and polyunsaturated and saturated FA associated with the "poor diet" were not specified, and *trans* FA were not singled out as an individual risk factor.

A similar study by van Dam *et al*. (125) examined dietary fat and meat intake in relation to risk of type 2 diabetes in 42,504 male subjects initially free of diabetes, cardiovascular disease, and cancer. Diet was assessed by food frequency questionnaire. During 12 yr of follow-up, 1,321 new cases of type 2 diabetes were observed. Intakes of total fat and saturated fat were associated with a higher risk of type 2 diabetes. However, these associations disappeared after adjustment for body mass index. Also, intakes of oleic acid, *trans* FA, long-chain n-3 FA, and α-linolenic acid were not associated with diabetes risk after multivariate adjustment. Results of this study with men were not consistent with the Salmeron *et al*. study (122) reporting *trans* FA to be positively associated and PUFA negatively associated with risk of type 2 diabetes in women.

Trans FA in relation to macular degeneration. Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment and blindness in the United States and other developed countries. Two recent studies from the same institution that evaluated a possible connection between dietary *trans* FA and type 2 diabetes also considered a possible relationship between *trans* FA and the risk of AMD.

One of these studies, by Seddon *et al*. (126), evaluated the relationship between intake of total fat and of specific FA, including *trans* FA, and risk of AMD in a case-control study at five U.S. clinical ophthalmology centers. Case subjects included 349 individuals (aged 55–80 yr) with the advanced neovascular stage of AMD diagnosed within 1 yr of their enrollment into the study. Control subjects included 504 individuals without AMD but with other ocular diseases. Higher consumption of vegetable fat, monounsaturated FA, PUFA, or linoleic acid was associated with an elevated risk of AMD. In contrast, higher consumption of saturated or *trans* FA was not associated with higher risk of AMD.

The association between AMD and higher intake of vegetable, monounsaturated, and polyunsaturated fats and linoleic acid was suggested to be related to snack foods containing products that adversely affect the blood vessels of the choroids or retina. The authors felt that such foods might also increase oxidative damage in the macula, which is susceptible to oxidation because of high oxygen tension in the presence of light exposure. Thus, some individual dietary fats, but not necessarily total fat consumption, might increase or decrease the risk for AMD.

Cho *et al*. (127) assessed a possible association between total fat and specific types of fat and AMD. This was a prospective study that at baseline included 42,743 women and 29,746 men, aged 50 yr or older with no diagnosis of AMD. During the 12-yr follow-up period, 567 subjects were diagnosed with AMD. The pooled (women and men) multivariate relative risk for the highest compared with the lowest quintile of total fat intake was 1.54, a value considered statistically significant. Unexplained was why the fourth quintile for fat intake had a relative risk equal to that of the first quintile and lower than that of the second and third quintiles. *Trans* fat intake was not related to risk of AMD, whereas α-linolenic acid intake was positively associated with risk of AMD. DHA, on the other hand, showed a slight inverse relationship with AMD, and more than four servings of fish per week was associated with a 35% lower risk of AMD compared with three or fewer servings per month.

In summary, one study has reported an association between *trans* FA intake and type 2 diabetes, however, a follow-up study found no such association. In view of no known functional or physiological connection between *trans* FA and type 2 diabetes, the reported association requires further testing. In the case of AMD, neither of two studies reported an association between dietary *trans* FA and risk of developing the condition. Considering the difficulty of conducting meaningful studies on effects of dietary fat on specific human disorders, such as type 2 diabetes and macular degeneration, it is not surprising that no adverse effects were found in the few studies reported in these areas.

DIETARY RECOMMENDATIONS REGARDING SATURATED AND *TRANS* FA

Current dietary recommendations by health professional organizations within and outside the United States (29,128–133) address intake of cholesterol-raising saturated and *trans* FA. These organizations are consistent in their recommendations of an upper limit of intake of saturated FA of 10% of energy and reduced intake of *trans* FA. Recommendations from U.S. organizations are summarized in Table 4, and those from organiza-

TABLE 4
Dietary Recommendations for Saturated and *trans* FA: U.S. Organizations[a]

Organization	Saturated FA	*trans* FA
American Heart Association	<7% energy (population)	<1% energy (population)
Adult Treatment Panel III of the National Cholesterol Education Program	<7% energy (those at risk)	Keep intake low
Health and Human Services/US Department of Agriculture	<10% energy (population)	Low as possible
Institute of Medicine of the National Academy of Sciences	Low as possible	Low as possible

[a]Current dietary recommendations of U.S. health professional organizations for saturated and *trans* FA.

tions in Europe and Japan, in Table 5. The consistency of these recommendations provides the basis for food manufacturers in the United States and globally to consider ways to decrease or eliminate *trans* FA from their food products.

FOOD INDUSTRY EFFORTS TO REPLACE OR REDUCE TRANS FA

Food manufacturers are using or developing basically four technological options to reduce or eliminate *trans* FA in their products. These options include: (i) modification of the hydrogenation process; (ii) use of interesterification; (iii) use of fractions high in solids from natural oils; and (iv) use of trait-enhanced oils. Information on each of these topics was presented at a recent American Oil Chemists' Society symposium on *trans* fat (134). Some approaches may be used in combination with others. Discussion of each option follows.

Modification of the hydrogenation process. Certain food products, such as spreads, margarines, and shortenings, require oils with specific levels of solids at different temperatures to achieve desired functionality. Solids normally come from either *trans* FA (resulting from hydrogenation) or saturated FA. Modifying the conditions of hydrogenation (e.g., pressure, temperature, and catalyst) affects the FA composition of the resulting oil, including the amount of *trans* FA formed, and properties such as m.p. and solid fat content of the oil. It is possible to make equivalently performing low-*trans* fats by increasing the degree of hydrogenation, which reduces the level of *trans* FA but increases the level of saturated FA. This could increase the m.p. of the fat, which, in turn, might affect the sensory properties of fried foods. Such fats will differ analytically from fats hydrogenated to a lesser degree because saturates now contribute most of the solids. This is illustrated in Figure 6 indicating that with progressive hydrogenation (decreasing iodine

TABLE 5
Dietary Recommendations for Saturated and *trans* FA: Non-U.S. Organizations[a]

Organization	Saturated FA	*trans* FA
Health Council of The Netherlands	Low as possible. UL 10% energy	Low as possible. UL 1% energy
Health Canada	<10% energy	
Ministry of Agriculture, United Kingdom	<10% energy	<2% energy
Austria, Germany, Switzerland	<10% energy	
Japan	6-8% energy	
World Health Organization/Food and Agricultural Organization of the United Nations	<10% energy; <7% energy for high-risk groups	<1% energy

[a]Current dietary recommendations of non-U.S. health professional organizations for saturated and *trans* FA.

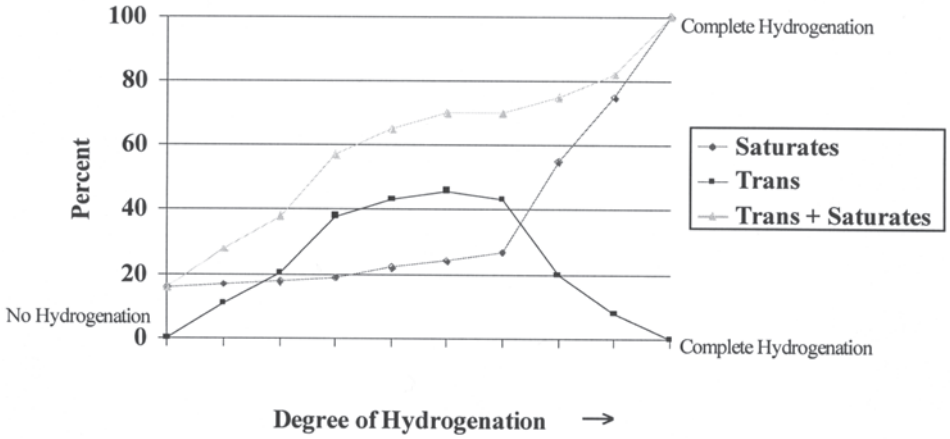


FIG. 6. Changes in percentages of *trans* and saturated FA with progressive hydrogenation of soybean oil. Progressive hydrogenation is indicated by decreasing iodine value. Corresponding levels of *trans* and saturated FA, expressed as percentage of total FA, are shown. The graph was provided by Bob Wainwright, Cargill, Inc.

value) of soybean oil, the content of *trans* FA increases to a maximum and then decreases essentially to zero. The corresponding level of saturated FA increases gradually until the content of *trans* FA is maximized and then increases rapidly.

Modification of the hydrogenation process can be used to prepare low-*trans* baking shortenings. The simple substitution of unhydrogenated (*trans*-free) fats for hydrogenated fats does not work well in baking applications. Some recipes for baked foods require a greater amount of saturated fat to replace a given amount of *trans* fat for the recipe to work (i.e., a one-to-one substitution of saturated fat for *trans* fat often does not produce foods of equivalent consumer acceptability).

Low- or zero-*trans* baking fats may have increased levels of stearic acid from the hydrogenation of α-linolenic, linoleic, and oleic acids, and also significant levels of palmitic acid for functionality. Palmitic acid may be provided by incorporating a high palmitic acid fat, such as cottonseed oil, into the shortening.

An increased level of stearic acid in a low- or zero-*trans* baking fat may offer nutritional benefits. Kelly *et al*. (135) reported that a high stearic acid diet reduced total, LDL-, and HDL-cholesterol levels in men compared with a baseline diet and also showed reduced factor VII coagulant activity and reduced mean platelet volume (both suggestive of decreased clotting tendency) compared with the baseline diet. In addition, an increased content of stearic acid in the serum FFA fraction has been associated with decreased risk of myocardial infarction (136).

Use of interesterification. The interesterification process involves the rearrangement (randomization) of the FA on the glycerol backbone of the fat (i.e., TG) in the presence of a chemical catalyst or an enzyme. Interesterification modifies the melting and crystallization behavior of the fat, thus producing fats with the desirable physical properties of *trans* fats but without *trans* FA. One current application of this process is in the production of *trans*-free or low-*trans* fats for margarine, spread, and shortening applications. Several human studies have shown no significant effects of interesterified fats on blood lipid parameters (137–140, summarized in 141).

In chemical interesterification, an unhydrogenated liquid vegetable oil (such as a salad oil) and a fully hydrogenated vegetable oil (called a "hardstock," which has a low iodine value and a low amount of *trans* FA) are mixed and heated. A typical blend may contain about 85% unhydrogenated oil and about 15% hardstock. Both components of the blend are essentially *trans*-free. A catalyst, commonly sodium methoxide, is added, and the interesterification process causes a random rearrangement of the FA on the glycerol backbone. It is important to note that in such blended fat products, the term "hydrogenated fat" or "partially hydrogenated oil" would appear on the ingredient statement, but the product would be very low in *trans* FA. Therefore, the listing of "hydrogenated fat" or "partially hydrogenated oil" on an ingredient statement does not always mean that the product contains a significant level of *trans* fat, as has been erroneously indicated by the popular press.

A recently modified interesterification process uses an enzyme instead of sodium methoxide to catalyze the reaction. The enzyme is a heat-stable, 1,3-specific lipase derived from a microorganism and immobilized on a granulated silica matrix. Because the lipase is specific for the 1- and 3-positions of the TG, its use results in a fat that is partially randomized rather than being completely randomized, as occurs with use of sodium methoxide. One advantage of enzymatic interesterification is that there are no waste or by-product issues, for example, no catalyst residues that could be released into the environment.

Table 6 compares the FA composition of a typical shortening fat prepared by partial hydrogenation with a newly developed product made by enzymatic interesterification. The interesterified fat is low in *trans* FA and has a lower oleic acid content and higher levels of stearic, linoleic, and α-linolenic acids compared with the partially hydrogenated fat. Although the FA

TABLE 6
FA Composition (percentages) of Shortenings Made by Partial Hydrogenation and by Enzymatic Interesterification[a]

FA (%)	Partial hydrogenation[b] (PHSBO, PHCSO)	Enzymatic interesterification[c] (SBO, FHSBO)
16:0	10.4	10.8
18:0	15.5	21.9
18:1 *cis* 9	37.5	21.9
18:2 *cis* 9,12	4.80	39.8
18:3 *cis* 9,12,15	0.30	5.6
Total *trans*	30.0	2.5–3.5 typical 5.0 max
Total saturates	25.9	32.7

[a]FA compositions and m.p. are shown for two shortening products, one made by conventional partial hydrogenation (using a blend of partially hydrogenated soybean oil [PHSBO] and partially hydrogenated cottonseed oil [PHCSO]) and one made by enzymatic interesterification (using a blend of unhydrogenated soybean oil [SBO] and fully hydrogenated soybean oil [FHSBO]). Data were provided by Tom Tiffany, Archer Daniels Midland Co. (Decatur, IL).
[b]m.p. (°F/°C): 117.5/47.5.
[c]m.p. (°F/°C): 110.0/43.3.

compositions of the two products differ from each other, the functional characteristics for shortening applications (e.g., m.p.) of the two products are very similar. The low level of *trans* FA (typically 2.5–3.5%, with a maximum of 5.0%) in this interesterified fat may have resulted in part from deodorization and/or incomplete hydrogenation of the component hydrogenated oil. Nevertheless, considering the normal 12-g serving size for shortening, the product made by interesterification would qualify for a declaration of 0.5 g of *trans* fat per serving according to the FDA's final labeling rule. If this shortening were incorporated into a baked product, depending on the amount of fat per serving, the product may be able to declare 0 g of *trans* fat per serving.

Use of fractions high in solids. Fractions high in solids derived from natural oils, namely coconut, palm, and palm kernel oils, are not new to the food industry and have been components of functional ingredients for years. Many commercially available fractions come from palm and palm kernel oils. They can be used successfully either as single fractions or in combination with other fractions to meet specific needs. Examples of fractions from palm oil that can be used in preparing *trans*-free or low-*trans* shortenings and margarines include palm olein, palm mid-fractions, and palm kernel stearin. Such fractions can be blended with liquid oils, such as soybean and canola oils, to create either no-*trans* shortenings and margarines or low-*trans*, low-saturates shortenings and margarines.

Fractions high in solids usually are prepared by reducing the temperature of an oil sample so that a more saturated fraction solidifies and a more unsaturated fraction remains liquid. The solid fraction is then physically separated from the liquid fraction by filtration or centrifugation. Liquid fractions, sometimes referred to as "olein fractions," are high in oleic acid and also are good sources of antioxidants, such as tocopherols and tocotrienols. Olein fractions frequently are used in Pacific Rim countries as salad oils and frying oils. Fractions with higher solids content may be incorporated into margarine or shortening fats.

Table 7 compares FA compositions of two no-*trans* shortenings and a no-*trans* margarine oil made with specific fractions from naturally solid vegetable oils such as palm or palm kernel oil. All of the products are *trans*-free, although there are differences in the levels of saturated monounsaturated, and polyunsaturated FA.

Use of trait-enhanced oils. Trait-enhanced oils generally fall into three categories: high-oleic acid oils, such as high-oleic sunflower and canola oils; mid-range oleic acid oils, such as mid-oleic sunflower and soybean oils; and low-linolenic acid oils, such as low-linolenic canola and soybean oils. (The term "low linolenic" commonly refers to an oil containing about 1–3% α-linolenic acid. Soybean oil typically contains about 7%, and canola oil, about 10% α-linolenic acid.) These types of oils are derived through traditional plant breeding or biotechnological methods. The high- and mid-range oleic acid oils are more stable oxidatively than traditional oils high in linoleic acid, such as soybean, corn, and sunflower oils. Low-linolenic acid oils have significantly reduced levels of oxidatively unstable α-linolenic acid. All of these trait-enhanced oils have good oxidative stability (i.e., a relatively high content of oleic acid

TABLE 7
FA Composition (percentages) and m.p. of No-*trans* Shortenings and a Margarine Oil Made with Fractions from Palm and/or Palm Kernel Oils[a]

Label declaration	No-*trans* shortening: Safflower, palm, and/or palm kernel oils[b]	No-*trans* shortening: Canola, palm, and/or palm kernel oils[c]	No-*trans* margarine oil: Canola, palm, and/or palm kernel oils[d]
TFA	0	0	0
SFA	35	23	26
MUFA	55	53	35
PUFA	10	24	39

[a]All products were made with either safflower oil or canola oil plus fractions of palm and/or palm kernel oils. Partial hydrogenation was not used in making these products. One shortening was made with safflower oil, and the other, with canola oil. Abbreviations are as follows: TFA, *trans* FA; SFA, saturated FA; MUFA, monounsaturated FA; and PUFA, polyunsaturated FA. Data were provided by Carl Heckel, Aarhus United USA Inc. (Newark, NJ).
[b]m.p. (°F/°C): 109/42.8.
[c]m.p. (°F/°C): 101/38.3.
[d]m.p. (°F/°C): 97/36.1.

TABLE 8
Typical FA Composition (percentages) of Trait-Enhanced Oils[a]

FA	Canola	High-oleic canola	Sunflower	Mid-oleic sunflower	High-oleic sunflower	Soybean	Low-linolenic soybean
16:0	5	4	7	5	4	11	10
18:0	2	2	4	4	4	4.5	5
18:1*c*9	61	74	14	58	79	25	28
18:2*c*9,12	21	12	74	32	11	51	56
18:3*c*9,12,15	10	4	<0.5	<0.5	<0.5	8	1

[a]Typical FA compositions of high-oleic canola oil, mid-oleic and high-oleic sunflower oils, and low-linolenic soybean oil are compared with their respective conventional oils. Data were provided by Bob Wainwright, Cargill, Inc. (Charlotte, NC).

and comparatively low levels of linoleic and α-linolenic acids), making them suitable for frying, spraying, beverages, and some bakery applications.

At present, the plant varieties that produce high-oleic and low-linolenic as well as some mid-oleic oils are not widely cultivated, and these oils are more expensive to produce than the corresponding polyunsaturated oils. Also, the high-oleic oils are liquid at room temperature and thus would not be particularly useful in spread and baking applications that require a relatively high solids content.

Table 8 shows typical FA compositions of several trait-enhanced oils. All have significant levels of oleic acid and comparatively lower levels of linoleic and α-linolenic acids. For salad oil and frying applications, these oils would not require hydrogenation and therefore would be *trans*-free.

The United Soybean Board (USB) has projected the future availability of low-linolenic soybean oil (having less than 3% α-linolenic acid) (142). Seed for low-linolenic soybeans has been developed by duPont (Wilmington, DE), Monsanto (St. Louis, MO), and Iowa State University (Ames, IA). For 2006, the USB expects 800,000 acres (320,000 hectares) to be planted, which likely would yield about 32 million bushels (870,000 metric tons) of soybeans and 320 million pounds (150,000 metric tons) of oil. By 2008, these totals are projected to be 4 million acres (1.6 million hectares), 160 million bushels (4.4 million metric tons) of soybeans, and 1.6 billion pounds (730,000 metric tons) of oil.

CURRENT STATUS OF USAGE OF ZERO- AND LOW-*TRANS* FATS

There is considerable interest in zero- and low-*trans* fats among food manufacturers, and current use of such products is increasing. For example, in frying applications, some restaurants and food service operations currently use unhydrogenated, *trans*-free oils. The use of such oils for frying will likely increase as greater quantities of trait-enhanced oils, such as high-oleic and mid-range-oleic oils, become available in the future. With regard to spreads and margarines, low-*trans* and *trans*-free products currently are marketed. Considering shortenings, in 2005 at least one *trans*-free product was being marketed. No safety or health issues are anticipated with the processes under consideration to reduce or eliminate *trans* FA from food products. Key challenges to manufacturers of zero- or low-*trans* fat products are to provide equivalent functionality at reasonable cost but without drastic increases in saturated FA content.

SUMMARY AND CONCLUSIONS

Dietary *trans* FA at sufficiently high levels have been found to increase LDL- and decrease HDL-cholesterol levels compared with diets high in *cis* monounsaturated or polyunsaturated FA. On the other hand, dietary *trans* FA have not been strongly related to cancer risk at specific sites or to other health conditions, including fetal growth and development, type 2 diabetes, and AMD. As a result of the effects of dietary *trans* FA on LDL- and HDL-cholesterol, most health professional groups have recommended reduced consumption of *trans* as well as saturated FA. Current efforts to replace or reduce *trans* FA in food products focus on: (i) modification of the hydrogenation process, (ii) use of interesterification, (iii) use of fractions high in solids from natural oils, and (iv) use of trait-enhanced oils.

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Effects of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA Isomers on Liver and Adipose Tissue Fatty Acid Profile in Hamsters

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ABSTRACT: The aim of the present study was to investigate the effect of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA on FA composition of TAG in epididymal adipose tissue and liver, and of hepatic phospholipids PL. Twenty-four Syrian Golden hamsters were randomly divided into three groups of eight animals each and fed semipurified atherogenic diets supplemented with either 0.5 g/100 g diet of linoleic acid or *cis*-9,*trans*-11 or *trans*-12,*cis*-9 CLA for 6 wk. Total lipids were extracted, and TAG and PL were separated by TLC. FA profile in lipid species from liver and adipose tissue, as well as in feces, was determined by GC. *Trans*-10,*cis*-12 CLA feeding significantly reduced linoleic and linolenic acids in TAG from both tissues, leading to reduced total PUFA content. Moreover, in the epididymal adipose tissue docosenoic and arachidonic acids were significantly increased. In liver PL, although no changes in individual FA were observed, total saturated FA (SFA) were decreased. No changes in TAG and PL FA profiles were induced by the *cis*-9,*trans*-11 CLA. TAG and PL incorporated *cis*-9,*trans*-11 more readily than *trans*-10,*cis*-12 CLA. This difference was not due to differential intestinal absorption, as shown by the analysis of feces. We concluded that only *trans*-10,*cis*-12 CLA induces changes in FA composition. Whereas increased PUFA content was observed in either liver or adipose tissue TAG, decreased SFA were found in liver PL. Incorporation of *cis*-9,*trans*-11 CLA in TAG is greater than that of *trans*-10,*cis*-12 CLA, but this is not due to differences in intestinal absorption.

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CLA has been shown to modify TAG metabolism. The size of adipose tissue, the main store of fat in the body, is reduced after CLA feeding. Although this body-fat lowering effect has been clearly observed in different rodent species and pigs (1–6), important differences in terms of responsiveness to CLA have been observed among species. For example, in mice, the most responsive rodent, several authors have found a very strong reduction in fat accumulation (lipodistrophy) after CLA feeding (7–9). Body fat reductions in hamsters and rats are more moderate.

CLA influences TAG metabolism not only in adipose tissue but also in the liver, an organ that plays a very important role in the control of lipid homeostasis. These effects have been shown to be species-specific as well: in mice CLA produces strong liver fat accumulation, leading to steatosis (7,10–12); in contrast, no changes (13,14) or even decreases (1,15,16) can be observed in liver TAG in rats and hamsters.

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Taking into account that in previous studies using the same experimental design as the present work quantitative changes brought about by CLA, namely a significant reduction in TAG accumulation either in adipose tissue or in liver (6,17–19), were observed, we were interested in assessing potential qualitative TAG changes. As a result, in this study the aim was to investigate the effect of dietary supplementation with the two main isomers of CLA, *cis*-9,*trans*-11 and *trans*-10,*cis*-12, on the FA profile of TAG in epididymal adipose tissue and liver in hamsters. Because the liver is a PL-rich organ, the influence on hepatic PL profile was also analyzed. In order to determine whether potential differences induced in FA profiles by both CLA isomers were due to different intestinal absorption rates, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers were quantified in feces.

EXPERIMENTAL PROCEDURES

Animals, diets, and experimental design. The experiment was conducted with 24 9-wk-old male Syrian Golden hamsters (105 ± 1 g) purchased from Harlan Ibérica (Barcelona, Spain), and took place following the institution's guide for the care and use of laboratory animals. The hamsters were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Gugguggiate, Italy) and placed in an air-conditioned room (22 ± 2°C) with a 12-h day-night rhythm. After a 6-d adaptation period, 24 hamsters were randomly divided into three groups of eight animals each and, over a period of 6 wk, fed semipurified atherogenic diets. CLA diets were enriched with 0.5 g/100 g diet of *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA, respectively. Control diet was supplemented with sunflower oil used as a source of linoleic acid (Tables 1 and 2). This work is a part of a bigger study in which the effects of CLA isomers on serum and liver cholesterol were assessed. For this purpose, atherogenic feeding has been recognized as a useful dietary tool. The experimental diets were freshly prepared once a week, gassed with nitrogen and stored at 0–4°C to avoid rancidity. DL-Methionine and cholesterol were supplied by Sigma (St. Louis, MO); wheat starch and cellulose by Vencasser (Bilbao, Spain); CLA, as free FA, by Natural Lipids Ltd. (Hovdebygda, Norway); sucrose and sunflower oil (the source of linoleic acid provided as TAG) were obtained from local markets; and palm oil was a generous gift from Agra-Unilever (Leioa, Spain). Vitamin and mineral mixes were formulated according to AIN-93 guidelines (20) and supplied by ICN Pharmaceuticals (Costa Mesa, CA). According to the manufacturer, the compositions

TABLE 1
Composition of Experimental Diets

Ingredients (g/kg)	Linoleic acid	*cis*-9,*trans*-11 CLA	*trans*-10,*cis*-12 CLA
Casein (90 g/100 g)	200	200	200
DL-Methionine	4	4	4
Sucrose	404	404	404
Wheat starch	200	200	200
Palm oil	100	100	100
Sunflower oil	8.2	—	—
cis-9,*trans*-11 CLA (90 g/ 100 g)	—	5.6	—
trans-10,*cis*-12 CLA (90 g/ 100 g)	—	—	5.6
Cholesterol	1	1	1
Cellulose	30	30	30
Mineral mix[a]	40	40	40
Vitamin mix[a]	11	11	11
Choline chloride salt	4	4	4

[a]Formulated according to the American Institute of Nutrition (AIN-93; Ref. 20).

of the CLA isomer test substances were as follows: *cis*-9,*trans*-11 CLA (92.5% *cis*-9,*trans*-11 CLA, 0.8% *trans*-10,*cis*-12 CLA, and 3.4% other CLA isomers) and *trans*-10,*cis*-12 CLA (92.5% *trans*-10,*cis*-12 CLA, 3.4% *cis*-9,*trans*-11 CLA, and 3.3% other CLA isomers).

All animals had free access to food and water. Food intake and body weight were measured daily. Feces were collected daily during the last week and stored at –80°C. For chromatographic analysis, the seven samples from each animal were pooled.

At the end of the experimental period, animals were killed under anesthesia (diethyl ether) with cardiac puncture. Epididymal adipose tissue and liver were dissected, weighed, and immediately dropped in liquid nitrogen. Samples were stored at –80°C until analysis.

FA profiles. Frozen samples of 0.5–1 g of tissues (liver and epididymal adipose) or feces were weighed, kept at 0°C in an ice bath, and homogenized in trichloromethane:methanol 2:1 (vol/vol) using a Potter Heidolph RZR 1 homogenizer (Schwabach, Germany). Samples were allowed to stand at room temperature for 1 h. *n*-Nonadecanoic acid (19:0) and trinonadecanoin were added as internal standards at the beginning of this process. After drying under nitrogen, the sample was dissolved in 1 mL of trichloromethane.

TAG and PL were separated by TLC on precoated silica plates (DC-Platten Kiesegel 60 F254, Merck, Frankfurt, Germany) with trichloromethane:*n*-hexane:glacial ethanoic acid 65:35:1 (by vol). Lipid classes were visualized with rhodamine 0.05% in absolute ethanol. Bands were identified by using standards for TAG (triolein) and PL (lecithin) on the basis of their retention factor (R_f), scraped off, recovered in trichloromethane, and filtered through glass wool. The obtained sample was dried under nitrogen and dissolved in 1 mL trichloromethane for methylation.

Methylation of TAG and *PL.* Sodium methoxide (500 μL in 0.5 M methanol) (Sigma-Aldrich, Steinheim, Germany) was added to lipid extracts to prepare FAME. The mixture was heated at 50°C for 10 min, and the reaction was stopped with 100 μL of glacial ethanoic acid. Water (5 mL) was added, and the required esters were extracted twice with *n*-hexane (5 mL).

TABLE 2
FA Composition of the Diet (mg/g Diet)

	Linoleic acid	*cis*-9, *trans*-11 CLA	*trans*-10, *cis*-12 CLA
12:0	0.46 ± 0.02	0.50 ± 0.05	0.49 ± 0.04
14:0	1.37 ± 0.04	1.49 ± 0.13	1.48 ± 0.12
16:0	35.91 ± 1.07	38.36 ± 3.25	37.17 ± 3.07
16:1n-7	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
18:0	4.17 ± 0.13	4.35 ± 0.43	4.13 ± 0.33
18:1n-9	25.56 ± 0.69	25.23 ± 1.91	24.62 ± 2.06
18:1n-7	1.11 ± 0.02	1.06 ± 0.21	1.06 ± 0.07
18:2n-6	14.89 ± 0.43	11.37 ± 0.99	11.08 ± 0.88
18:3n-3	0.13 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
20:0	0.12 ± 0.02	0.08 ± 0.01	0.09 ± 0.01
22:0	0.12 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
cis-9,*trans*-11	ND	5.26 ± 0.27	0.20 ± 0.01
trans-10,*cis*-12	ND	0.23 ± 0.01	5.15 ± 0.11
Total	83.14 ± 2.50	87.99 ± 6.89	85.71 ± 6.69

ND, not detectable.

TABLE 3
FA Composition (mg/g Tissue) of Hepatic TAG of Hamsters Fed Experimental Diets for 6 wk

	Linoleic acid	*cis*-9,*trans*-11 CLA	*trans*-10,*cis*-12 CLA	*P* value
14:0	0.08 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.475
16:0	1.28 ± 0.12	1.43 ± 0.15	1.09 ± 0.02	0.379
16:1n-7	0.16 ± 0.02	0.18 ± 0.03	0.12 ± 0.01	0.103
18:0	0.16 ± 0.02	0.18 ± 0.02	0.14 ± 0.01	0.502
18:1n-9	1.86 ± 0.15	2.12 ± 0.24	1.57 ± 0.07	0.186
18:1n-7	0.12 ± 0.01	0.14 ± 0.02	0.11 ± 0.01	0.308
18:2n-6	0.94 ± 0.06[a]	0.87 ± 0.09[a]	0.50 ± 0.03[b]	0.007
18:3n-3	0.013 ± 0.001[a]	0.010 ± 0.001[a]	0.005 ± 0.001[b]	0.007
20:0	0.012 ± 0.002	0.010 ± 0.001	0.007 ± 0.001	0.087
22:0	0.023 ± 0.003	0.019 ± 0.002	0.015 ± 0.002	0.076
20:4n-6	0.062 ± 0.012	0.061 ± 0.015	0.041 ± 0.004	0.421
cis-9,trans-11 CLA	ND	0.081 ± 0.012[a]	0.006 ± 0.001[b]	0.001
trans-10,cis-12 CLA	ND	0.007 ± 0.002[b]	0.015 ± 0.001[a]	0.001
Total	4.71 ± 0.38[a]	5.19 ± 0.59[a]	3.66 ± 0.13[b]	0.04

Values are means ± SEM. [a,b]Mean values within a row with different superscripts were significantly different; ND, not detectable.

The hexane layer was dried over anhydrous sodium sulphate (Merck, Frankfurt, Germany) and filtered with a #1 Whatman filter paper. The solvent was removed under reduced pressure on a rotatory evaporator and dissolved in 500 µL of *n*-heptane containing 50 ppm of BHT as stabilizer, before the GC analysis.

Analysis by GC. Samples with FAME were transferred to GC vials and analyzed with a Hewlett Packard HP6890 gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a FID, split/splitless injection port, an HP Chemstation software data system and an HP7673 autosampler (Hewlett Packard). The analytical column was a fused silica capillary SP-2380 column (100 m × 0.32 mm i.d., 0.20 µm film thickness) from Supelco (Bellafonte, PA). The oven temperature was initially programmed at 150°C (for 4 min) and raised to 220°C at a 4°C/min rate, then held for 25 min. Injection (1 µL) was run in split (40:1) mode. Helium was the carrier gas at constant flow (1 mL/min) and make-up gas for the FID. The injector was maintained at 200°C and detector at 250°C. FAME were identified by comparison of retention times with standards (Sigma, St. Louis, MO). Pure CLA isomers were purchased from Matreya (Matreya Inc., Pleasant GAP, PA).

Statistical analysis. Results are presented as means ± SEM. Statistical analysis was performed using SPSS 11.0 (SPSS Inc. Chicago, IL). Data were analyzed by one-way ANOVA followed by Newman-Keuls *post-hoc* test. Statistical significance was identified at the $P < 0.05$ level.

RESULTS

FA profile in liver. Table 3 shows the TAG FA profile. Total FA content in TAG was significantly reduced by *trans*-10,*cis*-12 CLA feeding but not by *cis*-9,*trans*-11 CLA. This CLA isomer did not induce changes in FA composition with the exception of its incorporation. In contrast, linoleic acid and linolenic acid were significantly reduced by *trans*-10,*cis*-12 CLA feeding, leading to reduced total PUFA content (53.5% lower than the linoleic group) (Fig. 1A). When the sums of saturated FA (SFA), monounsaturated FA (MUFA), and PUFA were expressed as the percentage of total FA quantified in TAG, a significant increase in SFA and a decrease in PUFA were observed in *trans*-10,*cis*-12 CLA–fed animals. With regard to *cis*-9,*trans*-11, a weaker but significant decrease in the percentage of PUFA was found (Fig. 1B). CLA feeding did not modify 16:0/16:1 n-9 ratio (7.95 ± 0.94 for LA group, 8.36 ± 1.76 for *cis*-9,*trans*-11 CLA group, and 9.16 ± 0.66 for *trans*-10,*cis*-12 CLA group; $P = 0.141$) nor 18:0/18:1 n-9 ratio (0.087 ± 0.010 for LA group, 0.086 ± 0.014 for *cis*-9,*trans*-11 CLA group, and 0.089 ± 0.006 for *trans*-10,*cis*-12 CLA group; $P = 0.878$). Incorporation of both CLA isomers into the liver TAG of CLA-exposed hamsters was evident.

The changes observed in TAG FA profile were not reflected in liver PL (Table 4). In this lipid species, no changes in total FA content were observed, and none of the FA was significantly modified by CLA isomers. Despite the lack of changes in individual FA, the sum of total SFA was significantly reduced by *trans*-10,*cis*-12 CLA (Fig. 2A). When the sums of SFA, MUFA, and PUFA were expressed as the percentage of total FA quantified in PL, no significant changes were found (Fig. 2B). The 16:0/16:1 n-9 and 18:0/18:1 n-9 ratios were not modified by CLA isomer feeding. Incorporation of both CLA isomers into the liver PL was markedly greater than that observed in hepatic TAG. The *cis*-9,*trans*-11 isomer was present in greater amount than *trans*-10,*cis*-12 CLA.

FA profile in epididymal adipose tissue. TAG FA profile is shown in Table 5. Total FA content in TAG was not modified. *Trans*-10,*cis*-12 CLA feeding significantly reduced linoleic acid and linolenic acid and increased docosenoic and arachi-

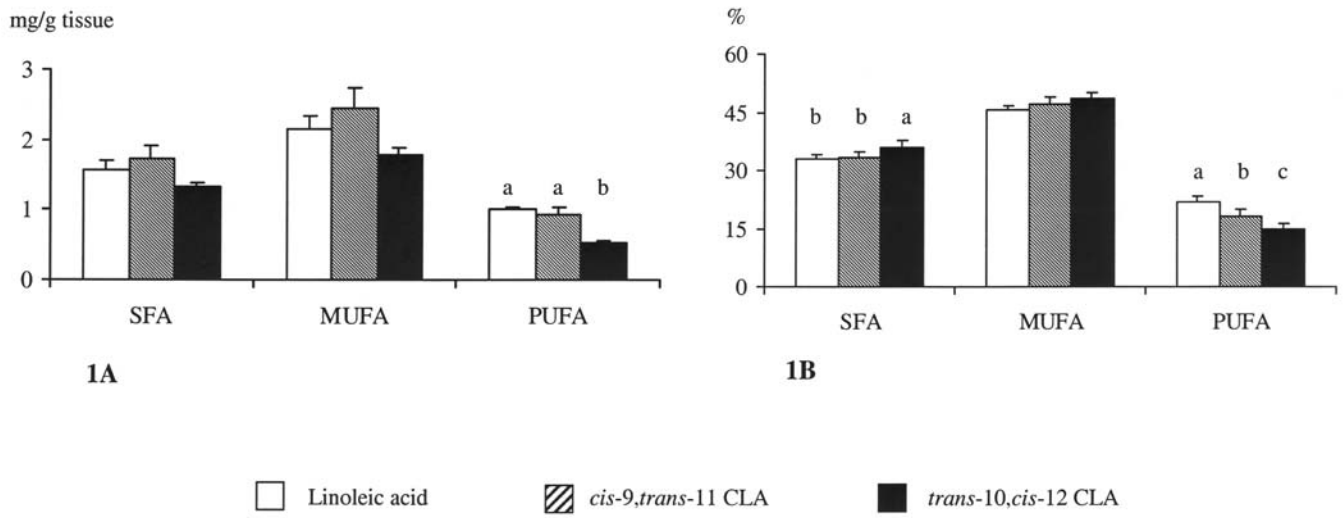

FIG. 1. Total SFA, MUFA, and PUFA in liver TAG of hamsters fed atherogenic diets supplemented with 0.5 g/100 g diet of linoleic acid, *cis*-9,*trans*-11 or *trans*-12,*cis*-9 CLA, respectively for 6 wk, expressed as mg/g tissue and as percentage of total FA. Values are means ($n = 8$) with their standard errors depicted by vertical bars. [a,b,c]Mean values with unlike superscript letters were significantly different ($P < 0.05$).

donic acids. No changes in FA profile were induced by the *cis*-9,*trans*-11. The sum of PUFA was significantly reduced in *trans*-10,*cis*-12 CLA–fed animals (Fig. 3A). Expressing SFA, MUFA, and PUFA as the percentage of total FA quantified in TAG, significant increases in SFA and MUFA, and a significant decrease in PUFA were observed after *trans*-10,*cis*-12 CLA feeding (Fig. 3B). As well as in liver TAG, CLA feeding did not modify 16:0/16:1 n-9 ratio (4.17 ± 0.49 for LA group, 4.73 ± 0.56 for *cis*-9,*trans*-11 CLA group, and 4.59 ± 0.61 for *trans*-10,*cis*-12 CLA group; $P = 0.194$) nor 18:0/18:1 n-9 ratio (0.058 ± 0.03 for LA group, 0.061 ± 0.004 for *cis*-9,*trans*-11 CLA group, and 0.063 ± 0.004 for *trans*-10,*cis*-12 CLA group; $P = 0.06$). Incorporation of both CLA isomers into the adipose tissue TAG was evident and sharply higher than in liver TAG. As in liver TAG and PL, the *cis*-9,*trans*-11 isomer was present in greater amount than the *trans*-10,*cis*-12.

CLA isomer content in feces. The amount of *cis*-9,*trans*-11 CLA in feces of hamsters fed the diet enriched with this isomer was 0.38 mg/g. That of *trans*-10,*cis*-12 CLA in animals fed the diet enriched with this isomer was 0.43 mg/g. Taking these values and daily food intake into account, the percentages of absorption were as follows: 99.5% for *cis*-9-*trans*-11 CLA and 98.3% for *trans*-10,*cis*-12 CLA. No significant differences were found among these values.

TABLE 4
FA Composition (mg/g Tissue) of Hepatic Phospholipids of Hamsters Fed Experimental Diets for 6 wk

	Linoleic acid	*cis*-9,*trans*-11 CLA	*trans*-10,*cis*-12 CLA	*P* value
12:0	0.70 ± 0.18	0.72 ± 0.29	0.71 ± 0.26	0.948
14:0	10.41 ± 3.72	11.16 ± 3.66	7.19 ± 2.65	0.829
16:0	28.84 ± 1.38	24.90 ± 1.58	23.95 ± 3.76	0.193
16:1n-7	2.82 ± 0.67	2.92 ± 0.69	2.94 ± 0.26	0.497
18:0	5.92 ± 0.86	8.49 ± 1.23	4.84 ± 0.87	0.148
18:1n-9	12.13 ± 2.23	17.65 ± 4.37	13.54 ± 2.57	0.816
18:1n-7	1.06 ± 0.24	1.16 ± 0.12	1.09 ± 0.24	0.983
18:2n-6	1.66 ± 0.24	3.50 ± 1.12	2.31 ± 0.27	0.578
18:3n-3	ND	ND	ND	
20:0	0.83 ± 0.25	1.17 ± 0.31	1.18 ± 0.47	0.717
22:0	0.56 ± 0.18	0.74 ± 0.17	0.64 ± 0.27	0.753
22:1n-11	0.86 ± 0.34	0.96 ± 0.36	1.21 ± 0.58	0.867
20:4n-6	1.16 ± 0.32	1.20 ± 0.44	1.75 ± 1.29	0.899
cis-9,*trans*-11 CLA	ND	1.13 ± 0.39	0.62 ± 0.26	0.462
trans-10,*cis*-12 CLA	ND	0.29 ± 0.10	0.88 ± 0.27	0.917
Total	66.82 ± 4.09	71.97 ± 7.22	62.17 ± 3.79	0.691

Values are means ± SEM. ND, not detectable.

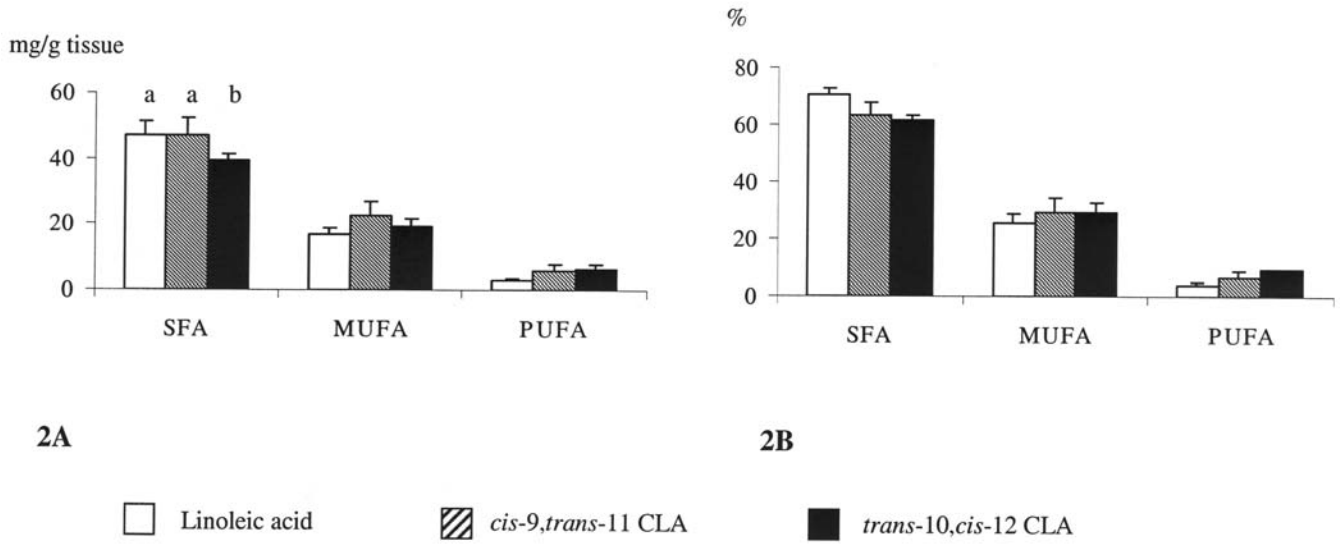

FIG. 2. Total SFA, MUFA, and PUFA in liver PL of hamsters fed atherogenic diets supplemented with 0.5 g/100 g diet of linoleic acid, *cis*-9,*trans*-11 CLA, or *trans*-12,*cis*-9 CLA for 6 wk, expressed as mg/g tissue and as percentage of total FA. Values are means ($n = 8$) with their standard errors depicted by vertical bars. [a,b]Mean values with unlike superscript letters were significantly different ($P < 0.05$).

DISCUSSION

Changes in FA composition. Several studies have set out to analyze the effect of CLA feeding, either as a CLA mixture or as individual isomers, on FA composition in different lipid species, such as TAG, PL, and cholesterol esters, in various organs and tissues. These studies have been carried out using not only rodent species such as mouse (22–28), rat (29–33), and hamster (1), but also in pigs (34–37) and humans (39–42).

In all these studies FA composition was analyzed semiquantitatively: the content of each FA was expressed as a percentage of total FA. In the present work our objective was to analyze changes in FA profile induced by each of the two main isomers of CLA administered separately, by measuring quantitatively the amount of each FA in the selected lipid species, liver TAG, liver PL, and adipose tissue TAG.

Substitution of linoleic acid for *trans*-10,*cis*-12 CLA in the diet resulted in important changes in FA profile in TAG. In liver TAG, linoleic and linolenic acids were significantly decreased, leading to reduced total PUFA content. No changes in other FA

TABLE 5
FA Composition (mg/g Tissue) of TAG in Epididymal Adipose Tissue of Hamsters Fed Experimental Diets for 6 wk

	Linoleic acid	*cis*-9,*trans*-11 CLA	*trans*-10,*cis*-12 CLA	*P* value
12:0	0.70 ± 0.04	0.83 ± 0.09	0.67 ± 0.05	0.363
14:0	9.80 ± 0.37	10.77 ± 0.58	11.15 ± 0.75	0.418
16:0	175.69 ± 8.68	193.57 ± 9.77	191.20 ± 10.14	0.421
16:1n-7	42.16 ± 1.91	40.91 ± 1.5	41,69 ± 2.98	0.722
18:0	15.44 ± 0.37	16.74 ± 1.02	17.23 ± 0.96	0.189
18:1n-9	257.50 ± 12.89	273.76 ± 13.71	273.39 ± 13.96	0.811
18:1n-7	17.02 ± 0.96	17.37 ± 0.77	16.91 ± 0.41	0.595
trans-18:1n-9	0.51 ± 0.03	0.53 ± 0.05	0.49 ± 0.03	0.878
trans-18:1n-7	0.52 ± 0.03	0.58 ± 0.04	0.50 ± 0.03	0.298
18:2n-6	140.95 ± 4.84[a]	140.44 ± 7.50[a]	111.22 ± 4.71[b]	0.007
18:3n-3	2.62 ± 0.10[a]	2.92 ± 0.22[a]	1.67 ± 0.07[b]	0.001
20:0	0.38 ± 0.02	0.42 ± 0.03	0.46 ± 0.05	0.251
22:0	0.22 ± 0.05	0.25 ± 0.03	0.31 ± 0.03	0.239
22:1n-11	0.09 ± 0.01[b]	0.08 ± 0.01[b]	0.17 ± 0.03[a]	0.032
20:4n-6	0.13 ± 0.02[b]	0.14 ±0.02[b]	0.29 ± 0.05[a]	0.022
cis-9,*trans*-11 CLA	ND	9.90 ± 0.40[a]	1.13 ± 0.08[b]	0.001
trans-10,*cis*-12 CLA	ND	0.13 ± 0.01[b]	5.17 ± 0.39[a]	0.001
Total	684.69 ± 20.73	702.06 ± 31.81	670.46 ± 32.93	0.610

Values are means ± SEM. [a,b]Mean values within a row with different superscript were significantly different. ND, not detectable.

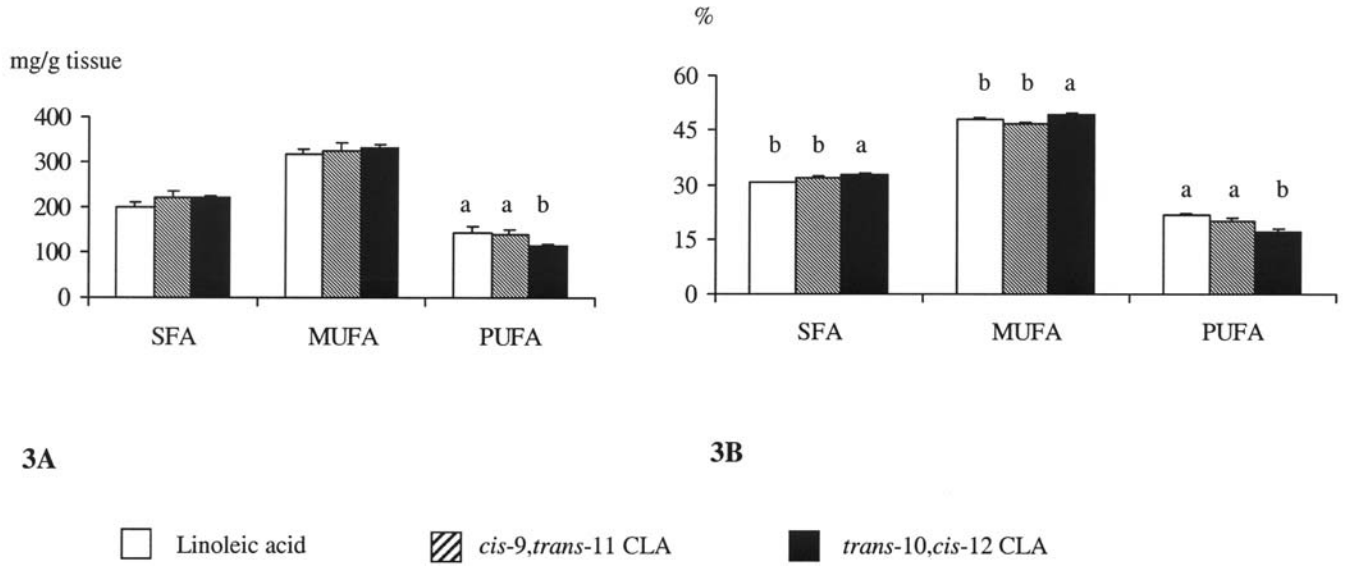

FIG. 3. Total SFA, MUFA, and PUFA in epididymal adipose tissue TAG of hamsters fed atherogenic diets supplemented with 0.5 g/100 g diet of linoleic acid, *cis*-9,*trans*-11 CLA, or *trans*-12,*cis*-9 CLA for 6 wk, expressed as mg/g tissue and as percentage of total FA. Values are means ($n = 8$) with their standard errors depicted by vertical bars. [a,b]Mean values with unlike superscript letters were significantly different ($P < 0.05$).

were observed. The same effect was found in epididymal adipose tissue TAG, but in this case a significant increase in docosenoic and arachidonic acids was also observed. The other CLA isomer, *cis*-9,*trans*-11, did not modify individual FA amounts either in liver TAG or in adipose tissue TAG.

Reductions in PUFA content, either in adipose tissue or in liver, have also been observed by other authors, although in these studies the amounts were expressed as percentages of total FA (23,25,38). With regard to arachidonic acid, several papers have shown a significant reduction of this FA in adipose tissue TAG from abdominal (29,37), perirenal (32), and retroperitoneal (27) anatomical locations in different animals species. In contrast, two studies performed in epididymal adipose tissue revealed a significant increase in arachidonic acid content (23,27). Our study, also carried out in the epididymal depot, is in good accord with these two works. Taking all these results into account, it could be hypothesized that the effects of CLA on TAG FA profile, or at least on arachidonic FA content, in adipose tissue depend on the anatomical location. Results concerning the effects of CLA on liver TAG arachidonic acid content are less consistent in the literature. Whereas Belury and Kempa-Steczko (22) reported a significant increase in mice by using 0.5% CLA and a significant decrease with higher doses, De Deckere *et al.* (1) did not find significant changes in hamsters fed *trans*-10,*cis*-12 CLA. Our results are in line with the latter study.

Some groups of researchers have reported a significant reduction in stearoyl-CoA desaturase (Δ9 desaturase) expression and/or activity induced by CLA (43–47). Moreover, significant increases in 16:0/16:1 n-9 and 18:0/18:1 n-9 ratios, two Δ9 desaturation indexes, have been found by some authors (29,30,34). In the present study, these two ratios were not modified in TAG in liver or in adipose tissue. These results suggest that CLA has no effect on this enzyme activity and agree with those observed by other authors (25,32,27,37). It seems that the effects of CLA on Δ9 desaturase are more evident when evaluating the activity or the expression of the enzyme than when the indirect indexes are calculated. Perhaps the ratios 16:0/16:1 n-9 and 18:0/18:1 n-9 are not as conclusive as believed. When the amount of 18:1 n-9 is high in the diet, as in the present experiment where oleic acid represents 28–30% of total FA in TAG (Table 2), and leads to significant accumulation of this FA in body lipids, small changes in the amount of this FA due to changes in Δ9 desaturase activity may not be evident.

Total FA content in liver TAG was significantly decreased in hamsters fed the *trans*-10,*cis*-12 CLA diet. This result is in good accord with the reduction in TAG content previously observed in hamsters fed the *trans*-10,*cis*-12 CLA isomer and measured by using a spectrophotometric method (19).

When total amounts of SFA, MUFA, and PUFA were expressed as percentages of total FA, SFA were increased in liver and adipose tissue TAG, MUFA were increased in adipose tissue TAG, and PUFA were reduced in liver and adipose tissue TAG by *trans*-10,*cis*-12 CLA feeding. When results are expressed in this way it is important to be careful not to give the wrong message. It should be pointed out that, despite the increased percentage of SFA, total amount of these FA was not actually enhanced. This important issue is seen only when a quantitative GC is used for FA profile analysis.

Liver PL were less sensitive than TAG to *trans*-10,*cis*-12 CLA feeding; no significant changes were observed in individual FA. By pooling FA according to their saturation index, important differences between liver TAG and liver PL were observed. Whereas TAG showed a decrease in PUFA, PL showed a decrease in SFA.

Incorporation of CLA isomers into tissues. The present results show that TAG incorporated *cis*-9,*trans*-11 more readily than *trans*-10,*cis*-12, the isomer responsible for body and liver fat reduction in hamsters (1,6,17–19). This pattern of accumulation was observed independently of the tissue analyzed (liver or adipose tissue). The selectivity in CLA isomer incorporation to TAG was in agreement with other studies in rodents (23,26,29,30,32).

The difference in CLA isomer accumulation was not due to differences in intestinal absorption because, taking into consideration CLA intake and fecal CLA excretion, similar percentages of absorption for both isomers were calculated. Thus, the results suggest a selective uptake of CLA isomers by tissues or a more efficient metabolism of *trans*-10,*cis*-12, leading to a reduced amount available to be stored. Martin *et al.* (48) found that the *trans*-10,*cis*-12 isomer was preferentially driven through the beta-oxidation pathway compared to the *cis*-9,*trans*-11 homologue. Moreover, Sébedio *et al.* (30) reported that the conversion of *trans*-10,*cis*-12 in long-chain metabolites is higher than that of *cis*-9,*trans*-11.

As expected, TAG deposition being largely greater in adipose tissue than in liver, dietary CLA isomers were incorporated into TAG mainly in adipose tissue and to a lesser extent in liver. Moreover, when the amount of CLA isomers was expressed as mg/g of TAG, the incorporation of both isomers was again greater in adipose tissue than in liver: 1.25 mg/g *cis*-9,*trans*-11 in liver TAG and 10.10 mg/g in adipose tissue TAG from hamsters fed this isomer, and 0.31 mg/g *trans*-10,*cis*-12 and 5.27 mg/g in adipose tissue TAG from hamsters fed this isomer.

With regard to liver PL, *cis*-9,*trans*-11 isomer was incorporated to a greater extent than *trans*-10,*cis*-12 isomer, similarly to that found in liver TAG. CLA isomer amounts in PL were greater than those in liver TAG. In the case of *cis*-9,*trans*-11 this difference was not due to a preferential incorporation into PL (1.56% in TAG and 1.57% in PL), but rather to the fact that PL content in a healthy liver is greater than TAG content. In contrast, in the case of *trans*-10,*cis*-12 isomer a more ready incorporation into PL (1.36%) than into TAG (0.40%) was observed.

It seems surprising to have found the *cis*-9,*trans*-11 isomer in *trans*-10,*cis*-12–fed hamsters and *vice versa*. A possible explanation is that the supplements, although highly enriched with one isomer, also contained about 6% of the other; the amounts of the of the minority CLA isomer found in tissue lipids probably arise from this small amount in the supplement.

In conclusion, this study shows that only *trans*-10,*cis*-12 CLA induces changes in FA composition. Whereas increased PUFA content was observed in either liver TAG or adipose tissue TAG, decreased SFA were found in liver PL. Incorporation of *cis*-9,*trans*-11 CLA in TAG is greater than that of the *trans*-10,*cis*-12 isomer, but this is not due to differences in intestinal absorption.

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Highly Unsaturated Fatty Acid Synthesis in Marine Fish: Cloning, Functional Characterization, and Nutritional Regulation of Fatty Acyl Δ6 Desaturase of Atlantic Cod (*Gadus morhua* L.)

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ABSTRACT: This study reports the cloning, functional characterization, tissue expression, and nutritional regulation of a Δ6 fatty acyl desaturase of Atlantic cod (*Gadus morhua*). PCR primers were designed based on the sequences of conserved motifs in available fish desaturases and used to isolate a cDNA fragment from cod liver, with full-length cDNA obtained by rapid amplification of cDNA ends. The cDNA for the putative desaturase was shown to comprise 1980 bp, including a 261-bp 5′-UTR, a 375-bp 3′-UTR, and an ORF of 1344 bp that specified a protein of 447 amino acids. The protein sequence included three histidine boxes, two transmembrane regions, and an N-terminal cytochrome b_5 domain containing the heme-binding motif HPGG, all characteristic of microsomal fatty acyl desaturases. The cDNA displayed Δ6 desaturase activity in a yeast expression system. Quantitative real-time PCR assay of gene expression in cod showed that the Δ6 desaturase gene was expressed highly in brain, to a slightly lesser extent in liver, kidney, intestine, red muscle, and gill, and at much lower levels in white muscle, spleen, and heart. The expression of the Δ6 desaturase gene did not appear to be under significant nutritional regulation, with levels in liver and intestine being barely altered in fish fed a vegetable oil blend, in comparison with levels in fish fed fish oil. This was reflected in enzyme activity, as hepatocytes or enterocytes showed very little highly unsaturated FA biosynthesis activity irrespective of diet. Further studies are required to determine why the Δ6 desaturase appears to be barely functional in cod under the conditions tested.

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Fish are the only major dietary source for humans of n-3 highly unsaturated FA (HUFA), EPA (20:5n-3), and DHA (22:6n-3) (1). With traditional fisheries declining, aquaculture supplies an increasing proportion of the fish in the human food basket (2). However, the current high use of fish oil (FO), derived from feed-grade marine fisheries, in aquaculture feeds is not sustainable, and will constrain growth of aquaculture activities (3,4). Vegetable oil (VO), a sustainable alternative to FO, can be rich in C_{18} PUFA such as linoleic (18:2n-6) and α-linolenic (18:3n-3) acids, but lack the n-3 HUFA abundant in FO (5). The extent to which fish can convert C_{18} PUFA to $C_{20/22}$ HUFA varies with species, and is associated with their capacity for fatty acyl desaturation and elongation (6). Marine fish are unable to produce EPA and DHA from 18:3n-3 at a physiologically significant rate (5) due to apparent deficiencies in one or more enzymes in the desaturation/elongation pathway (7,8). Thus, flesh FA compositions in marine fish fed VO are characterized by increased levels of C_{18} PUFA and decreased levels of n-3 HUFA, compromising their nutritional value to the human consumer (9,10).

Until depletion of the commercial stocks in the 1990s, Atlantic cod (*Gadus morhua* L.) was the most valued food fish obtained from the North Atlantic (11). In recent years significant progress has been made in the culture of cod and the life cycle has been closed, allowing production independent of wild fisheries (12). The large and highly developed market for cod, along with the high market price and the decreasing quotas set to preserve wild stocks, has greatly increased interest in cod culture in recent years (13–15). However, the establishment of large-scale, sustainable cod culture will require solutions to several nutritional issues including broodstock and larval nutrition and replacement of dietary FO with alternatives in on-growing diets (16–18).

Our overall aim is to determine what regulates HUFA biosynthesis in fish and how it can be optimized to enable fish to make effective use of dietary VO. Recently, fatty acyl desaturases, critical enzymes in the pathways for the biosynthesis of the long-chain $C_{20/22}$ HUFA from shorter-chain C_{18} PUFA, have been cloned from several teleosts (19,20). The cDNA for Δ6 and Δ5 desaturases have been cloned from Atlantic salmon (*Salmo salar* L.) (21,22) whereas, in contrast, only Δ6 desaturase cDNA have been cloned from marine fish (23,24). The expression levels of both Δ6 and Δ5 desaturases in salmon were shown to be upregulated in fish fed VO, in comparison with

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Abbreviations: FO, fish oil; HUFA, highly unsaturated FA (carbon chain length ≥ C20 with ≥ 3 double bonds); ORF, open reading frame; Q-PCR, quantitative real-time polymerase chain reaction; RACE, rapid amplification of cDNA ends; $SCMM^{-uracil}$, Saccharomyces cerevisiae minimal medium minus uracil; UPM, universal primer mix; UTR, untranslated region; VO, vegetable oil.

levels in fish fed FO (25,26). The specific objectives of the study described here were to investigate FA desaturation and the regulation of the HUFA synthetic pathway in Atlantic cod. Thus, we describe the cDNA cloning, functional characterization, and tissue distribution of a fatty acyl desaturase of cod, and report the effects of nutrition on the expression of fatty acyl desaturase and elongase genes in cod fed diets containing either FO or a blend of VO.

MATERIALS AND METHODS

Cloning of putative fatty acyl desaturase from cod. Liver tissue was obtained from Atlantic cod fed for 3 mon with the VO diet described below. Total RNA was extracted from liver using TRIzol® reagent (Invitrogen Ltd., Paisley, UK). 3'-RACE cDNA was synthesized using MMLV reverse transcriptase (Promega, Madison, WI) primed by the oligonucleotide Not1polyT, 5'-GATAGCGCCGCGTTTTTTTTTTTTTTTV-3'. 5'-RACE-cDNA was synthesized using the SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA). Available fish desaturase sequences were aligned to enable the design of the degenerate forward primer, 5'-CARCAYCAYGCNAARCCNAA-3', and reverse primer, 5'-RAANARRTGYTCDATYTG-3'. These were used for PCR isolation of a cod desaturase cDNA fragment. PCR amplification was performed using Thermoprime plus DNA polymerase (ABgene, Surrey, UK) under the following touchdown PCR conditions: initial denaturation at 95°C for 1 min, 10 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 30 s (–1.5°C /cycle), and extension at 72°C for 90 s, then 6 cycles of denaturation at 95°C for 15 s, annealing at 52°C for 30 s, and extension at 72°C for 90 s, followed by 24 cycles of denaturation at 95°C for 15 s, annealing at 51°C for 30 s, and extension at 72°C for 90 s. The PCR products were cloned into pBluescript KS II$^+$ vector (Stratagene, La Jolla, CA). The nucleotide sequences were determined by standard dye terminator chemistry using an Applied Biosystems ABI-377 DNA sequencer following the manufacturer's protocols (Applied Biosystems, Foster City, CA). Following isolation of a cDNA fragment flanked by the above primers, the specific forward primer 5'-GCCGATGAACATAGACCACG-3' was designed for 3' RACE PCR together with primer Not1PolyT under the following conditions: initial denaturation at 95°C for 1 min, 25 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min. The reverse primer, 5'-GGTGTTGGTGGTGATAGGG-CAT-3', was used in conjunction with forward universal primer mix (UPM), long and short, 5'-CTAATACGACTCACTATAG-GGCAAGCAGTGGTATCAACGCAGAGT-3' and 5'-CTAA-TACGACTCATATAGGGC-3', to perform 5' RACE PCR. Amplification involved an initial step at 95°C for 1 min, then 70°C for 3 min, and 5 cycles of denaturation at 95°C for 30 s, annealing at 60°C (–1°C/cycle) for 30 s, and extension at 72°C for 2 min, followed by 24 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min. Nucleotide sequences were determined as above, and the 3' and 5' RACE PCR fragment sequences were aligned to assemble the full nucleotide sequence of the cod putative desaturase cDNA using BioEdit version 5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, Raleigh, NC).

Heterologous expression of desaturase ORF in Saccharomyces cerevisiae. Expression primers were designed for PCR cloning of the cod putative desaturase cDNA ORF. The forward primer, 5'-CGGAATTCAAGCTTAAGATGGGAGGTGGAGGGCA-3', contained a *HindIII* site (underlined), and the reverse primer, 5'-GCTCTAGACTCGAGTCACTTATGGAGATAAGCATC-3', contained an *XhoI* site (underlined). PCR was performed using high-fidelity DNA polymerase (Roche Diagnostics Ltd., Lewes, East Sussex, UK), following the manufacturer's instructions. Amplification involved an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min. Following PCR, the DNA fragments were restricted and ligated into the similarly digested yeast expression vector pYES2 (Invitrogen Ltd., Paisley, UK). Ligation products were then used to transform Top10F' *E. coli* competent cells (Invitrogen Ltd.), which were screened for the presence of recombinants. Transformation of the yeast *S. cerevisiae* (strain InvSc1) with the recombinant plasmids was carried out using the S.c.EasyComp Transformation Kit (Invitrogen Ltd.). Selection of yeast containing the desaturase/pYES2 constructs was on *S. cerevisiae* minimal medium minus uracil (SCMM$^{-uracil}$). Culture of the recombinant yeast was carried out in SCMM$^{-uracil}$ broth as described previously (19), using galactose induction of gene expression. Each culture was supplemented with one of the following PUFA substrates: α-linolenic acid (18:3n-3), linoleic acid (18:2n-6), eicosatetraenoic acid (20:4n-3), dihomo-γ-linolenic acid (20:3n-6), docosapentaenoic acid (22:5n-3), or docosatetraenoic acid (22:4n-6). PUFA were added to the yeast cultures at concentrations of 0.5 mM (C_{18}), 0.75 mM (C_{20}), or 1 mM (C_{22}), as uptake efficiency decreases with increasing chain length. Yeast cells were harvested, washed, dried, and lipid extracted by homogenization in chloroform/methanol (2:1, vol/vol) containing 0.01% BHT as antioxidant, as described previously (19). FAME were prepared, extracted, purified by TLC, and analyzed by GC, all as described below. The proportion of substrate FA converted to the longer-chain FA product was calculated from the gas chromatograms as 100 × [product area/(product area + substrate area)]. Unequivocal confirmation of FA products was obtained by GC–MS of the picolinyl derivatives as described in detail previously (19).

Fish and diets. Atlantic cod were from a stock held at the Institute of Aquaculture (University of Stirling, Stirling, UK) and were fed a commercial fishmeal and oil-based diet prior to the trial. Fish with a mean body weight of 87 ± 5 g were stocked randomly (at 20 fish/tank) into two 1.5-m tanks of 400-L capacity supplied with recirculated seawater (37 ppt) at a constant temperature of 10°C at 400 L/h and were subjected to a photoperiod regime of 12 h light:12 h dark. Fish were fed at a rate of 1.5% biomass/d in two portions (9 am and 2 pm) for 3

TABLE 1
Formulations and proximate compositions of experimental diets[a]

	Fish oil	Vegetable oil
Fishmeal[b]	670	670
Soyabean meal[c]	86	86
Wheat[d]	90	90
Vitamin mix[e]	10	10
Mineral mix[f]	24	24
Carboxymethylcellulose	15	15
Fish oil[g]	100	—
Rapeseed oil[h]	—	40
Linseed oil[i]	—	40
Palm oil[j]	—	20
Choline chloride	4	4
Antioxidant mix[k]	1	1
Proximate composition		
Protein	50.0 ± 0.1	50.0 ± 0.2
Lipid	15.7 ± 0.1	15.3 ± 0.1
Ash	10.4 ± 0.1	10.4 ± 0.1
Fibre	1.6 ± 0.2	1.6 ± 0.3
Moisture	12.1 ± 0.0	13.3 ± 0.1

[a]Formulation in $g.Kg^{-1}$. Proximate compositions are percentages of total diet and are means ± S.D. (n=3).
[b]LT94, Norsemeal Ltd., London, UK.
[c]Cargill, Swinderbury, UK.
[d]J.D. Martin, Tranent, UK.
[e]Supplied (per kg diet): KH_2PO_4, 22g; $FeSO_4.7H_2O$, 1.0g; $ZnSO_4.7H_2O$, 0.13g; $MnSO_4.4H_2O$, 52.8 mg; $CuSO_4.5H_2O$, 12 mg; $CoSO_4.7H_2O$, 2 mg.
[f]Supplied (mg/kg diet): ascorbic acid, 1000; myoinositol, 400; nicotinic acid, 150; calcium pantothenate, 44; all-rac-α-tocopheryl acetate, 40; riboflavin, 20; pyridoxine hydrochloride, 12; menadione, 10; thiamine hydrochloride, 10; retinyl acetate, 7.3; folic acid, 5; biotin, 1; cholecalciferol, 0.06; cyanocobalamin, 0.02.
[g]FOSOL, Seven Seas Ltd., Hull, UK.
[h]Tesco, Cheshunt, UK.
[i]Croda, Hull, UK.
[j]United Plantations Bhd, Jenderata Estate, Teluk Intan, Malaysia.
[k]Dissolved in propylene glycol and contained (g/L): butylated hydroxy anisole, 60; propyl gallate, 60; citric acid, 40. All the other ingredients were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK.

mon before sampling. The diets were manufactured in-house, and differed only in the oil added to the basal mix (Table 1). In one diet, FO was the only added oil, and in the other diet FO was replaced by a VO blend containing rapeseed, linseed, and palm oils in a 2:2:1 ratio (Table 2). The diets were formulated to meet all the known nutritional requirements of marine fish (27). The experiment was conducted in accordance with British Home Office guidelines regarding research on experimental animals.

After 90 d, the fish weighed 258 ± 21 g, and there was no significant difference between weights of fish on different dietary treatments. Twelve fish per dietary treatment were sampled. Six were used for the preparation of hepatocytes from liver and preparation of enterocytes from pyloric ceca. Tissue samples were collected from the other six fish, with liver and cecal tissue collected from all six fish, and brain, heart, kidney, gill, spleen, adipose tissue, white muscle, and red muscle collected from three of these fish for RNA extraction and gene expression studies, and liver, ceca, and white muscle collected from four fish for lipid and FA analyses. All tissue samples were frozen immediately in liquid nitrogen and stored at –80°C prior to further analyses.

Cod tissue RNA extraction and quantitative real-time PCR (Q-PCR). Total RNA extraction was performed as described above. Five micrograms of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase first-strand cDNA synthesis kit (Promega UK, Southampton, UK). Gene expression of the fatty acyl Δ6 desaturase and FA elongase (28) genes in tissues from cod fed the different diets was studied by Q-PCR. The PCR primers were designed according to the Δ6 desaturase (accession no. DQ054840), and published elongase (accession no. AY660881) cDNA sequences. For the Δ6 desaturase, the forward and reverse primers were 5'-CCCCAGACGTTTGTGTCAG-3' and 5'-CCTGGATTGTTGCTTTGGAT-3', respectively. For the elongase, the forward and reverse primers were 5'-TGATTTGTGTTCCAAATGGC-3' and

TABLE 2
Fatty acid composition of experimental diets for Atlantic cod (*Gadus morhua*)[a]

	Fish oil	Vegetable oil
14:0	6.5	2.6
16:0	15.4	14.3
18:0	1.9	2.5
Total saturated[b]	24.7	20.2
16:1n-7[c]	6.0	3.1
18:1n-9	11.7	26.4
18:1n-7	2.6	2.5
20:1n-9[d]	11.1	5.7
22:1n-11[e]	14.9	6.2
Total monoenes	46.4	43.9
18:2n-6	2.7	10.3
20:4n-6	0.5	0.2
Total n-6 PUFA[f]	3.7	10.7
18:3n-3	1.3	13.6
18:4n-3	2.8	0.9
20:4n-3	0.5	0.2
20:5n-3	8.4	4.0
22:5n-3	0.9	0.3
22:6n-3	10.8	6.1
Total n-3 PUFA[g]	24.8	25.1
Total PUFA[h]	28.9	35.9

[a]Results are presented as percentages of total fatty acids by weight and are means of analyses of two samples.
[b]Totals contain 15:0, 17:0, 20:0 and 22:0 at up to 0.4%.
[c]Contains n-9 isomer present at up to 0.1%.
[d]Contains n-11 and n-7 isomers present at up to 0.4%.
[e]Contains n-9 and n-7 isomers at up to 1.0%.
[f]Totals contain 20:2n-6, 20:3n-6 and 22:5n-6 at up to 0.2%.
[g]Totals contain 20:3n-3 present at up to 0.1%.
[h]Contains C_{16} PUFA present at up to 0.4%.

5'-CTCATGACGGGAACCTCAAT-3', respectively. PCR product sizes were 181 and 219 bp, respectively. Amplification of cDNA samples and DNA standards was carried out using SYBR Green PCR Kit (Qiagen, Crawley, West Sussex, UK) and the following conditions: 15 min denaturation at 95°C, 45 cycles of 15 s at 94°C, 15 s at 55°C, and 30 s at 72°C. This was followed by product melt to confirm a single PCR product. Thermal cycling and fluorescence detection were conducted in a Rotor-Gene 3000 system (Corbett Research, Cambridge, UK), with expression of target genes quantified and normalized as described previously (29).

Lipid extraction and FA analyses. Total lipids of livers, pyloric ceca, flesh (white muscle), and diet samples were extracted by homogenization in chloroform/methanol (2:1, vol/vol) containing 0.01% BHT as antioxidant (30). FAME were prepared from total lipid by acid-catalyzed transesterification using 2 mL of 1% H_2SO_4 in methanol plus 1 mL toluene (31), and FAME extracted and purified as described previously (32). FAME were separated and quantified by GLC (Fisons GC8600, Fisons Ltd., Crawley, UK) using a 30 m × 0.32 mm capillary column (CP wax 52CB; Chrompak Ltd., London, UK). Hydrogen was used as carrier gas, and temperature programming was from 50°C to 180°C at 40°C/min and then to 225°C at 2°C/min. Individual methyl esters were identified by comparison to known standards and by reference to published data (33).

Activity of the HUFA biosynthesis pathway in hepatocytes and enterocytes. Hepatocytes and enterocytes were prepared from livers and pyloric ceca by collagenase treatment of chopped tissue and sieving through 100-μm nylon gauze essentially as described in detail previously for salmonid and sea bass tissues (26,34). Approximately twice as much cod liver tissue was used for digestion due to the high fat content and consequently lower yield of cells (35). Viability, as assessed by trypan blue exclusion, was > 95% at isolation and decreased by less than 5% over the period of the incubation for both cell types. The enriched enterocyte preparation was predominantly enterocytes although some secretory cells were also present. One hundred microliters of the hepatocyte and enterocyte suspensions were retained for protein determination according to the method of Lowry *et al.* (36) following incubation with 0.4 mL of 0.25% (wt/vol) SDS/1 M NaOH for 45 min at 60°C.

Five milliliters of either hepatocyte or cecal enterocyte suspension were dispensed into 25-cm^2 tissue culture flasks and

incubated at 20°C for 2 h with 0.3 μCi (~1 μM) [1-^{14}C]18:3n-3, added as a BSA complex (37). After incubation, cells were harvested, washed, and lipid extracted as described previously (26), and total lipid transmethylated and FAME prepared as described above. The FAME were separated by argentation (silver nitrate) TLC (38), located on the plate by autoradiography for 14 d, and quantified by liquid scintillation after being scraped from the plates as described previously (39).

Materials. [1-^{14}C]18:3n-3 (50–55 mCi/mmol) was obtained from NEN (Perkin Elmer LAS Ltd., Beaconsfield, U.K.). Eicosatetraenoic (20:4n-3), docosapentaenoic (22:5n-3), and docosatetraenoic (22:4n-6) acids (all >98–99% pure) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Linoleic (18:2n-6), α-linolenic (18:3n-3), and eicosatrienoic (20:3n-6) acids (all >99% pure), collagenase (type IV), FAF-BSA, BHT, and silver nitrate were obtained from Sigma-Aldrich Co. Ltd. (Poole, UK). TLC (20 × 20 cm × 0.25 mm) plates pre-coated with silica gel 60 (without fluorescent indicator) were purchased from Merck (Darmstadt, Germany). All solvents were HPLC grade and were from Fisher Scientific (Loughborough, UK).

RESULTS

Sequence analyses. The full length of the putative cod desaturase cDNA (mRNA), as determined by 5' and 3' RACE PCR, was shown to be 1980 bp, which included a 5'-UTR of 261 bp and a 3'-UTR of 375 bp. Sequencing revealed that the cDNA included an ORF of 1344 bp, which specified a protein of 447 amino acids (GenBank accession no. DQ054840). The protein sequence included all the characteristic features of microsomal fatty acyl desaturases, including two transmembrane regions, three histidine boxes, and an N-terminal cytochrome b_5 domain containing the heme-binding motif, HPGG (Fig. 1). Phylogenetic analysis comparing the cod desaturase sequence with desaturases from other fish species and human Δ5 and Δ6 desaturases clustered the cod most closely with tilapia, sea bream, and turbot, and more distantly from carp, zebrafish, and salmonids (Fig. 2). A pairwise comparison between fish and human desaturase sequences showed that the amino acid sequence predicted by the cod putative desaturase ORF showed greatest identity with that of gilthead sea bream Δ6 desaturase (82%), and 64% and 56% identity to the human Δ6 and Δ5 cDNA, respectively (Table 3).

Functional characterization. The FA composition of the yeast transformed with the vector alone showed the four main FA normally found in *S. cerevisiae*, namely 16:0, 16:1n-7, 18:0, and 18:1n-9, together with the exogenously derived FA (Fig. 3A and C). The most prominent additional peaks were observed in the profiles of transformed yeast grown in the presence of the Δ6 desaturase substrates, 18:2n-6 and 18:3n-3 (Fig. 3A–D). Based on GC retention time, and confirmed by GC-MS, the additional peaks associated with the presence of the cod desaturase cDNA were identified as 18:3n-6 (Fig. 3B) and 18:4n-3 (Fig. 3D), corresponding to the Δ6 desaturation products of 18:2n-6 and 18:3n-3, respectively. Approximately, 33.5% of 18:3n-3 was converted to 18:4n-3, and 17.5% of 18:2n-6 was converted to 18:3n-6 in yeast transformed with the cod desaturase. No additional peaks representing desaturated FA products were observed in the lipids of transformed *S. cerevisiae* incubated with 20:3n-6 or 20:4n-3 (Fig. 3E and F), indicating no Δ5 desaturase activity. Similarly, the cod desaturase cDNA did not express any Δ4 desaturase activity, as evidenced by the lack of additional peaks representing desaturated products of 22:5n-3 or 22:4n-6 (data not shown).

Fatty acyl desaturase and elongase gene expression in cod tissues. The fatty acyl Δ6 desaturase and PUFA elongase genes were expressed in all cod tissues examined. For the desaturase, the abundance of transcript was clearly greatest in brain, followed by liver, kidney, intestine, red muscle, and gill, and by much lower levels in white muscle, spleen, and heart (Fig. 4). The abundance of elongase transcript was very high in brain and gill, with lower levels in kidney, spleen, intestine, and heart, and a surprisingly low level of expression in liver.

Effect of diet on FA compositions of flesh, liver, and pyloric ceca. The FO diet contained around 25% total saturates, predominantly 16:0; approximately 46% total monoenes, with 26% as the long-chain monoenes 20:1 and 22:1; 3.7% n-6 PUFA, predominantly 18:2n-6; and almost 25% n-3 PUFA, with almost 20% as the n-3 HUFA 20:5n-3 and 22:6n-3 (Table 2). The VO diet provided slightly lower levels of total saturates and monoenes and a similar level of n-3 PUFA, but was characterized by increased proportions of 18:1n-9, 18:2n-6, and 18:3n-3, and decreased proportions of n-3 HUFA and long-chain monoenes. Therefore, the VO diet showed levels of 18:1n-9, 18:2n-6, and 18:3n-3 of around 26%, 10%, and 13%, respectively, whereas the combined level of 20:5n-3 and 22:6n-3 was reduced by half to 10% (Table 2).

The total lipid content of liver was greater than 50% of the wet weight, consistent with it being the major lipid storage organ in cod, whereas flesh had a low lipid content of 1%, and pyloric ceca contained around 3% total lipid (Table 4). Dietary VO had no significant effect on tissue lipid contents. The rank order of the major FA from total lipid of flesh from cod fed FO was 22:6n-3 > 16:0 > 20:5n-3 > 18:1n-9 > 20:1n-9 (Table 4). The changes in FA composition of the diets in response to replacement of FO with VO, described above, were reflected in the flesh FA compositions with increased proportions of 18:1n-9, 18:2n-6, and 18:3n-3, and decreased proportions of n-3 HUFA. Pyloric ceca showed a similar FA composition to flesh, with dietary VO having a very similar effect. However, consistent with the higher storage (neutral) lipid content, liver FA composition was characterized by higher levels of monounsaturated FA. Thus, the rank order of the major FA from total lipid of liver from fish fed FO was 18:1n-9 > 16:0 > 20:1n-9 > 22:6n-3 > 22:1 > 20:5n-3 > 16:1n-7 (Table 4). However, substitution of FO with VO resulted in similar changes to liver FA composition as in the other tissues, with increased proportions of 18:1n-9, 18:2n-6, and 18:3n-3, and decreased proportions of n-3 HUFA.

Effects of diet on the HUFA biosynthesis pathway in hepatocytes and enterocytes. Total activity of the HUFA biosynthe-

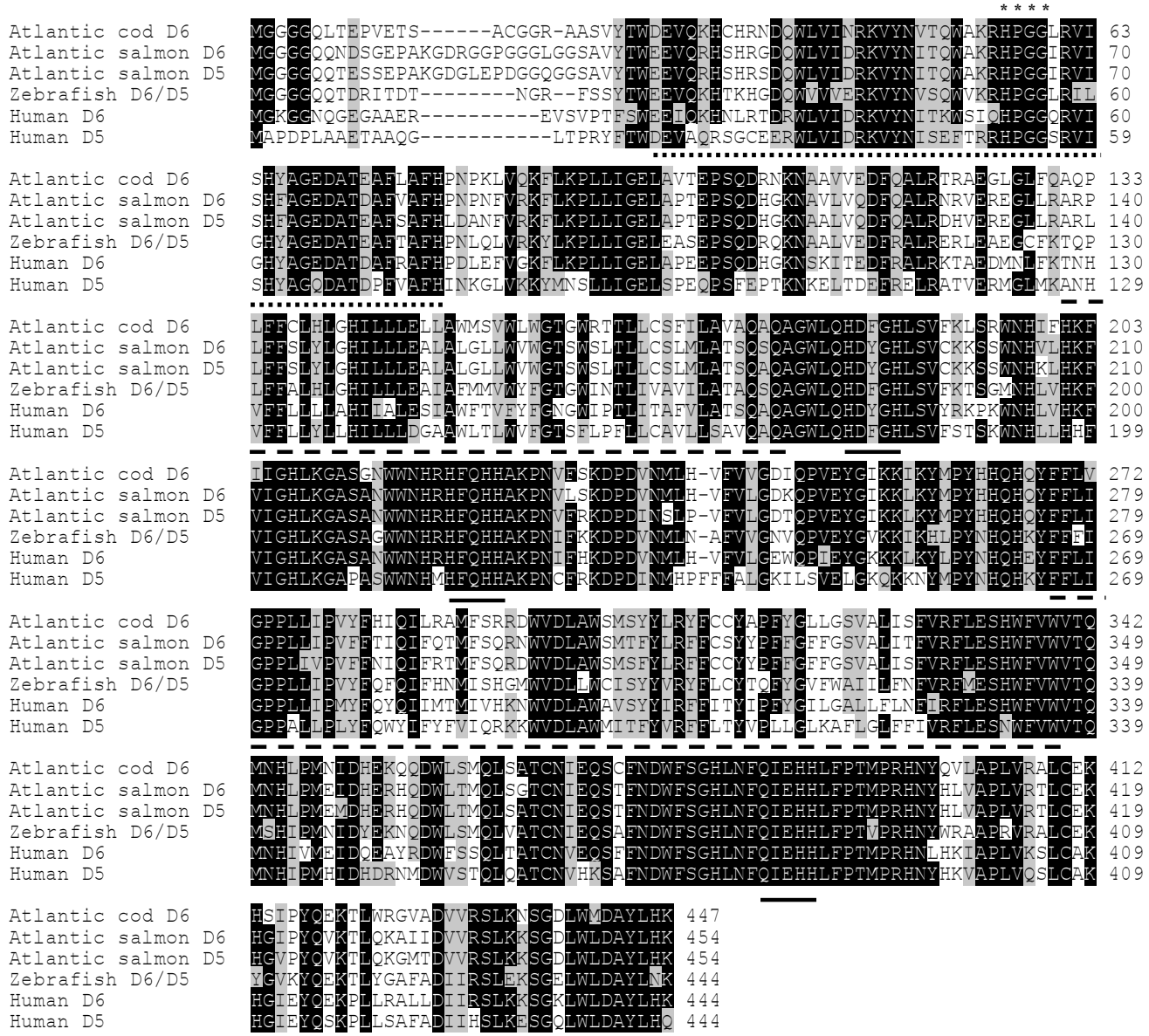


FIG. 1. Comparison of the deduced amino acid sequence of the Δ6 polyunsaturated fatty acyl desaturase from Atlantic cod with that of Δ6 and Δ5 desaturases from Atlantic salmon and human, and the Δ6/Δ5 bifunctional desaturase from zebrafish. Deduced amino acid sequences were aligned using ClustalX. Identical residues are shaded black and similar residues are shaded grey. Identity/similarity shading was based on the BLOSUM62 matrix, and the cutoff for shading was 75%. The cytochrome b_5–like domain is dot-underlined, the two transmembrane regions are dash-underlined, and the three histidine-rich domains are solid–underlined. The asterisks on the top mark the heme-binding motif, HPGG.

sis pathway as measured by the recovery of radioactivity in the summed desaturated products of [1-^{14}C]18:3n-3 was around 7-fold higher in enterocytes than in hepatocytes in cod (Fig. 5). In both cell types, the rate of synthesis of pathway products was unaffected by dietary treatment. The rate of the HUFA biosynthesis pathway in cod hepatocytes was between 40- and 120-fold lower than that observed in hepatocytes of Atlantic salmon smolts of similar size and assayed at the same time of year and fed similar diets (26) (Fig. 6). Similarly, the rate of the pathway in cod enterocytes was between 8- and 17-fold lower than that observed in salmon enterocytes. Furthermore, in contrast to the situation in cod, the rate of the HUFA biosynthesis pathway in salmon was significantly higher in both cell types from fish fed a VO blend compared with fish fed FO.

Effect of diet on expression of fatty acyl desaturase and elongase genes in liver and intestine. The effect of diet on the normalized expression of the Δ6 desaturase and PUFA elongase genes was determined in liver and intestine (pyloric ceca). The expression of both genes tended to be increased in terms of absolute copy number, but the data were characterized by a high degree of

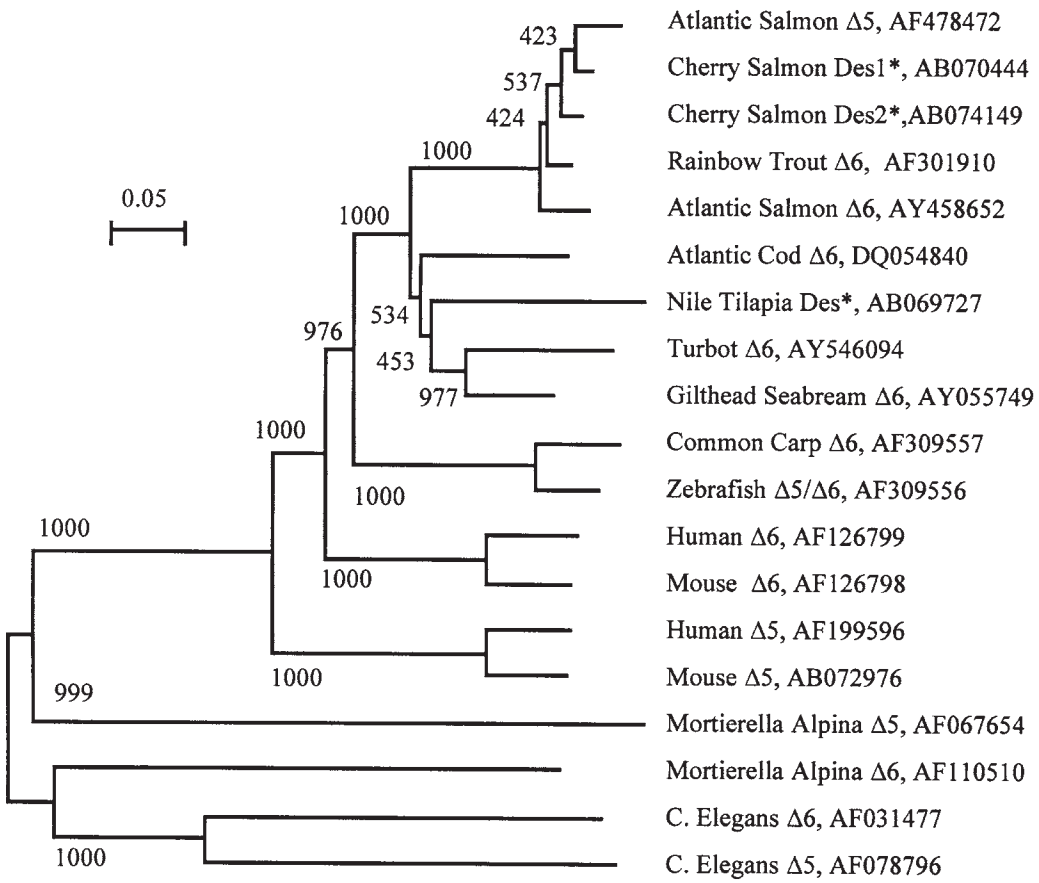


FIG. 2. Phylogenetic tree of cod Δ6 desaturase and desaturases from other fish species (Atlantic salmon, zebrafish, cherry salmon, rainbow trout, seabream, common carp, turbot, and tilapia), mammals (mouse and human), fungus (*Mortierella alpina*), and nematode (*Caenorhabditis elegans*). The tree was constructed using the neighbor-joining method (40) using *CLUSTALX* and *NJPLOT*. The horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. Sequences marked with an asterisk are not functionally characterized.

TABLE 3
Identity Matrix Showing the Results of a Pair-Wise Comparison Between the Amino Acid Sequences of Fish and Human Fatty Acyl Desaturases[a]

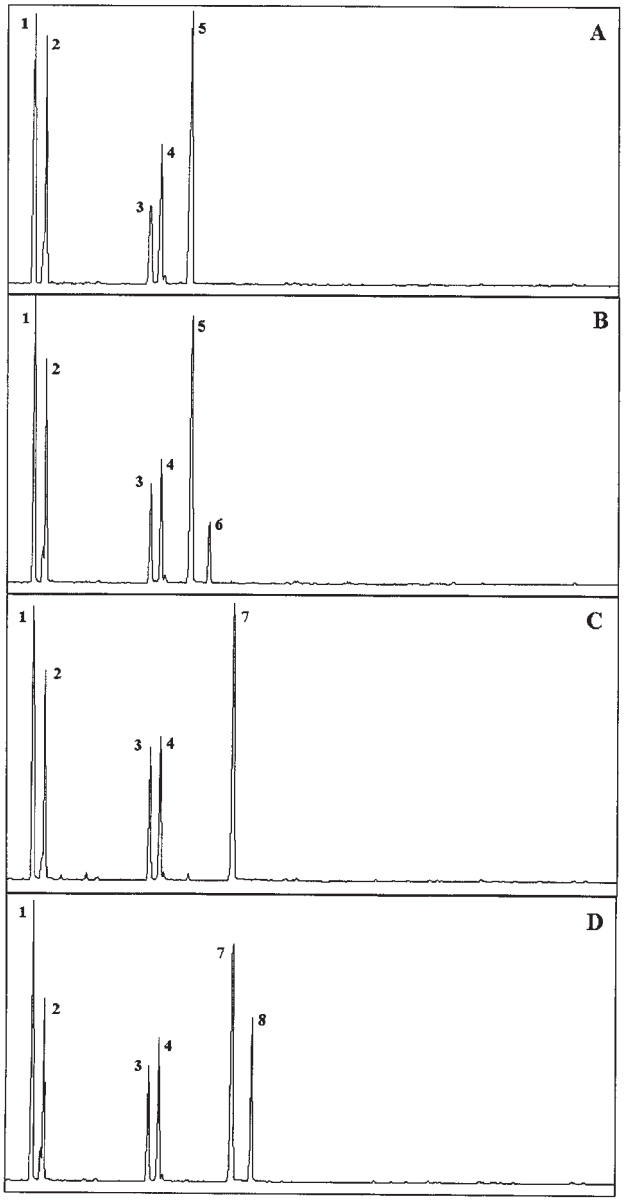
	Gilthead seabream Δ6	Turbot Δ6	Rainbow trout Δ6	Atlantic salmon Δ6	Atlantic salmon Δ5	Zebrafish Δ6/Δ5	Human Δ6	Human Δ5
Atlantic cod Δ6	82	77	76	75	76	70	64	56
Gilthead seabream Δ6		84	76	76	77	68	65	57
Turbot Δ6			72	72	73	68	62	56
Rainbow trout Δ6				94	92	65	65	57
Atlantic salmon Δ6					91	65	65	58
Atlantic salmon Δ5						64	63	57
Zebrafish Δ6/Δ5							65	56
Human Δ6								61

[a]Percentage of identical amino acid residues.

variation. Therefore, the only statistically significant effect of diet was increased expression of PUFA elongase in the liver (Fig. 7).

DISCUSSION

The study reported here revealed that Atlantic cod express a fatty acyl desaturase, and functional analysis in yeast confirmed the cod enzyme as a Δ6 desaturase. Comparing the protein sequence with sequences of a range of other desaturases of fish and human showed the cod Δ6 sequence to be more similar to the human Δ6 than to the human Δ5, but most similar to the Δ6 desaturases previously cloned from other marine fish, particularly gilthead sea bream (*Sparus aurata*) and turbot (*Psetta maximus*) (20,22). Phylogenetic analysis of the desaturase sequences reflected classical phylogeny, showing the cod (Paracanthopterygii; Gadiformes) branching from the Acanthopterygia (cichlids, perci-



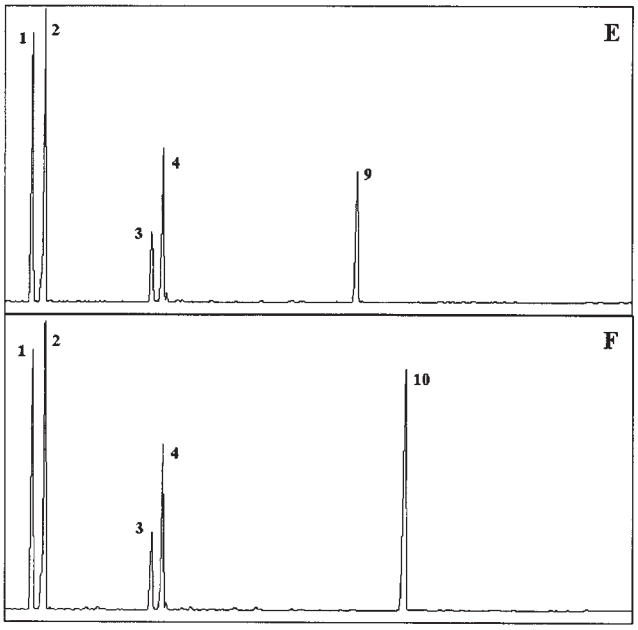


FIG. 3. Functional expression of the Atlantic cod putative fatty acyl desaturase in transgenic yeast (*Saccharomyces cerevisiae*). Panels A and C show the FA extracted from yeast transformed with pYES vector without insert and grown in the presence of 18:2n-6 and 18:3n-3. Panels B, D, E, and F show the FA compositions of yeast transformed with pYES vector containing the putative desaturase insert and grown in the presence of 18:2n-6, 18:3n-3, 20:3n-6, or 20:4n-3, respectively. The first four peaks in all panels are the main endogenous FA of *S. cerevisiae*, namely 16:0 (1), 16:1n-7 (2), 18:0 (3), and 18:1n-9 (with 18:1n-7 as shoulder) (4). Peak 5 in panels A and B and peak 7 in panels C and D are the exogenously added substrate FA, 18:2n-6 and 18:3n-3, respectively. Peaks 6 and 8 in panels B and D were identified as the resultant desaturated products, namely 18:4n-3 and 18:3n-6, respectively. Peaks 9 and 10 in panels E and F are the exogenously added substrate FA, 20:3n-6 and 20:4n-3, respectively. Vertical axis, FID response; horizontal axis, retention time.

formes, and pleuronectiformes) line, and further separated from both the carp and zebrafish (Ostariophysi; cyprinids), and salmonids (Salmoniformes; salmonidae) (42). Along with the cloning of Δ6 desaturase cDNA of sea bream and turbot, the work described has confirmed that marine fish have, and express, the gene required for the first step in the HUFA biosynthesis pathway, Δ6 desaturation, suggesting that any deficiencies in this pathway in marine fish would be at a further step such as chain elongation and/or Δ5 desaturation. This is consistent with biochemical data suggesting deficiencies in these steps in turbot and sea bream cell lines (7,8).

However, despite expressing an apparently active Δ6 desaturase, the activity of the HUFA biosynthesis pathway in both hepatocytes and enterocytes in cod was very low, and considerably lower than the activities measured in salmon hepatocytes and enterocytes (26,43). Indeed, the activities were so low that it was not possible to accurately quantify individual products, although in enterocytes, in which the activity was slightly higher, we were able to confirm that only Δ6 desaturated products (18:4n-3, 20:4n-3, and 22:4n-3) were observed on the autoradiographs. Furthermore, the majority product was 18:4, and so very little HUFA (defined as $\geq C_{20}$ and ≥ 3 double bonds) was actually produced, and neither EPA nor DHA were detected in cod. In contrast, functional expression in the yeast system had shown that the cod Δ6 had substantial enzymatic activity toward 18:3n-3. This perhaps highlights the known limitations of heterologous expression systems that use the endogenous yeast transcription system rather than the cod transcription machinery. It is also clear that it is essential that gene expression at the protein level should also be studied to confirm translation of the Δ6 desaturase gene in cod tissues. However, in the same yeast system, the salmon Δ6 gene gave over 60% conversion of 18:3n-3 (22), compared with 33% for the cod Δ6. Therefore, consistent with the salmon expressing higher HUFA synthesis activities, the salmon Δ6 was more active than the cod Δ6 in a comparative system, although this comparison is limited by the considerations mentioned above. Conversely, conversion of 18:2n-6 by the cod Δ6 exceeded that

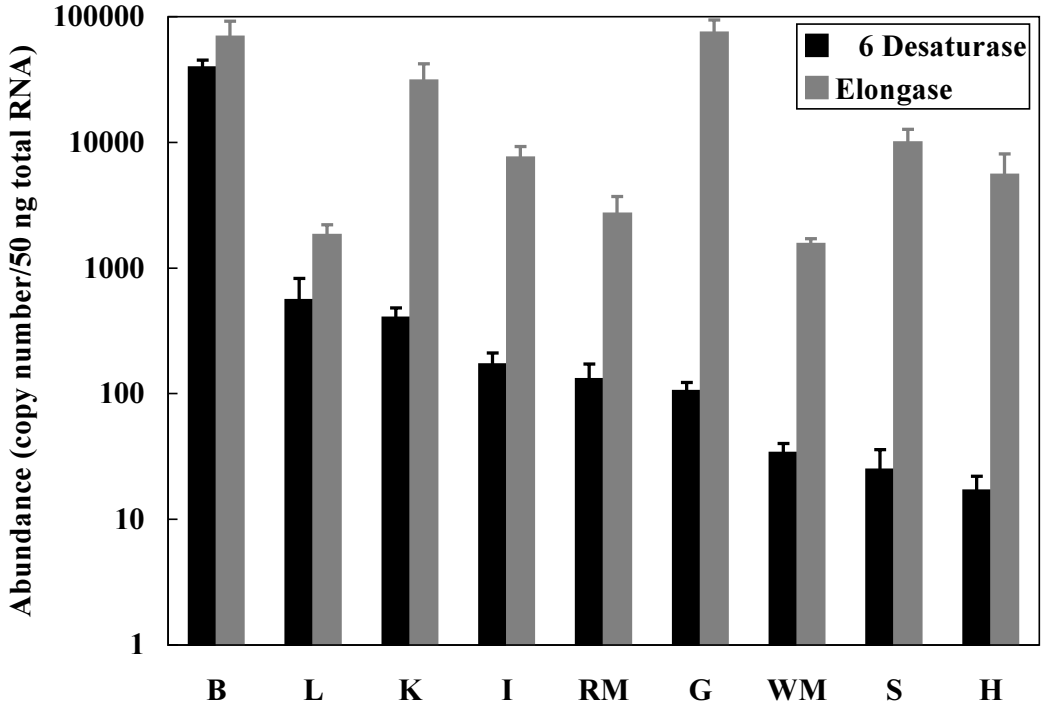


FIG. 4. Tissue distribution of fatty acyl Δ6 desaturase and PUFA elongase genes in Atlantic cod. Transcript (mRNA) copy number was determined by Q-PCR and normalized as described in the text. Results are expressed as means ± SEM (n = 4). L, liver; H, heart; G, gill; WM, white muscle; RM, red muscle; I, intestine; B, brain; A, adipose; S, spleen; K, kidney.

of the salmon Δ6 (18% *vs*. 14%) in the yeast expression system (22). This was interesting as it had previously been shown that the cod PUFA elongase was unique among the fish desaturase and elongase genes functionally characterized so far by being more active toward the n-6 substrate than the equivalent n-3 substrates (28). The reason for the cod genes being relatively more active toward n-6 substrates is unclear.

In addition to being considerably lower than in salmon, the rate of the HUFA biosynthesis pathway observed in cod was even lower than that observed in previous studies in other marine fish. The rate of desaturation of ^{14}C-18:3n-3 to all products in sea bass (*Dicentrarchus labrax*) was around 0.2 pmol.h^{-1}.mg protein^{-1} in hepatocytes and between 0.7 and 1.1 pmol.h^{-1}.mg protein^{-1} in cecal enterocytes, but this latter figure still represented only 2.1% of total radioactivity recovered, with 97.9% of total radioactivity recovered as the substrate [1-^{14}C]18:3n-3 (34). The activities in cod were around 10-fold lower at 0.02 and 0.15 pmol.h^{-1}.mg protein^{-1} in hepatocytes and enterocytes, respectively. It was perhaps noteworthy that the HUFA biosynthesis activities were higher in enterocytes than in hepatocytes in both sea bass and cod. This may be a real biological difference, as intestine is now acknowledged as a site of significant FA metabolism, at least in salmonids (44). However, it is possible that part of that difference may be due to the practical problems in isolating cells from such a lipid-rich tissue as cod liver. Despite using twice as much liver as ceca (in terms of wet weight), the cell yield of hepatocytes was very low and may have been a contributing factor. Problems of low yield from lipid-rich livers, and also of analyzing autoradiography data from very low activity samples (see above), were encountered in the previous study in sea bass (34).

Expression of the cod Δ6 desaturase was greatest in brain followed by liver, kidney, and intestine, whereas expression of the PUFA elongase was very high in brain and gill with a low level of expression in liver. In salmon, the expression of both Δ6 and Δ5 desaturases and the PUFA elongase were highest in intestine, liver, and brain (22). Mammalian Δ6 and Δ5 desaturases also show relatively high expression in liver and brain, as well as heart and kidney (45,46). A third desaturase gene in humans (FADS3) also shows highest expression in brain, heart, and liver (47). In contrast, intestine does not appear to be a site of high desaturase expression in mammals (48). In mouse, a PUFA elongase was expressed to the greatest extent in testis and liver followed by brain and kidney (49). In rat, two elongases were isolated: one, a PUFA elongase (rELO1), was expressed highly in lung and brain, whereas the other (rELO2), responsible for elongating saturated FA, was expressed only in liver (50). The tissue expression analysis in cod also showed that expression of PUFA elongase exceeded Δ6 desaturase expression. This was in contrast with the situation in salmon, where both Δ6 and Δ5 desaturase expression exceeded expression of PUFA elongase in all tissues (22). This may be a real biological difference between cod and salmon, but there may be other contributing factors. For example, in salmon, there are at least two other desaturase gene sequences in the genome (X. Zheng, unpublished data). The entire DNA gene sequences for

TABLE 4
Total Lipid Content and FA Composition of Total Lipid from Liver, Pyloric Caeca, and Flesh (white muscle) of Atlantic Cod Fed Diets Containing Fish Oil (FO) or a Vegetable Oil Blend[a] (VO)

	Liver		Pyloric caeca		Flesh	
FA	FO	VO	FO	VO	FO	VO
Lipid content	58.3 ± 3.1	52.7 ± 5.2	3.1 ± 0.4	3.2 ± 0.5	1.0 ± 0.2	1.0 ± 0.2
14:0	4.5 ± 0.4	2.2 ± 0.2*	2.9 ± 0.8	1.6 ± 0.2*	2.0 ± 0.4	1.0 ± 0.1*
16:0	14.6 ± 0.7	12.1 ± 0.5*	17.7 ± 0.6	15.2 ± 0.3*	18.2 ± 0.8	16.1 ± 0.9*
18:0	2.6 ± 0.5	2.9 ± 0.1	3.4 ± 0.4	4.0 ± 0.6	3.0 ± 0.2	3.5 ± 0.1*
Total saturated[b]	22.2 ± 0.8	17.5 ± 0.6*	24.4 ± 0.5	21.2 ± 0.6*	23.5 ± 0.7	20.8 ± 0.9*
16:1n-7[c]	6.6 ± 0.5	3.9 ± 0.4*	3.2 ± 0.8	2.3 ± 0.4	2.9 ± 0.6	1.6 ± 0.3*
18:1n-9	18.7 ± 0.7	31.8 ± 1.3*	11.5 ± 0.6	22.5 ± 2.3*	11.4 ± 1.8	17.7 ± 1.7*
18:1n-7	4.1 ± 0.4	3.5 ± 0.1	3.0 ± 0.1	2.7 ± 0.1	2.5 ± 0.2	2.3 ± 0.2
20:1n-9[d]	12.1 ± 0.9	7.1 ± 0.7*	6.7 ± 1.1	4.6 ± 0.2*	4.7 ± 1.2	2.9 ± 0.4*
22:1	8.4 ± 0.5	3.7 ± 0.4*	5.7 ± 2.0	3.1 ± 0.6	2.6 ± 1.3	1.4 ± 0.2
24:1n-9	0.6 ± 0.1	0.4 ± 0.0	1.9 ± 0.2	1.4 ± 0.3	0.7 ± 0.1	0.6 ± 0.0
Total monoenes	50.5 ± 0.6	50.4 ± 0.7	32.2 ± 4.3	36.7 ± 2.9	24.8 ± 5.0	26.5 ± 2.2
18:2n-6	3.7 ± 1.0	10.7 ± 0.2*	2.3 ± 0.3	8.6 ± 1.1*	2.9 ± 0.2	8.8 ± 0.6*
20:3n-6	0.5 ± 0.3	0.4 ± 0.4	0.2 ± 0.0	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	0.4 ± 0.0	0.2 ± 0.0*	1.7 ± 0.3	0.9 ± 0.2*	1.2 ± 0.2	0.8 ± 0.1*
Total n-6 PUFA[e]	5.1 ± 1.2	11.5 ± 0.5*	4.7 ± 0.2	10.4 ± 0.9*	4.7 ± 0.3	10.1 ± 0.6*
18:3n-3	1.3 ± 0.1	10.9 ± 0.9*	0.8 ± 0.2	7.4 ± 1.6*	1.0 ± 0.4	7.3 ± 1.2*
18:4n-3	2.4 ± 0.1	0.9 ± 0.1*	1.1 ± 0.3	0.5 ± 0.1*	1.2 ± 0.3	0.4 ± 0.0*
20:4n-3	0.6 ± 0.0	0.2 ± 0.0*	0.5 ± 0.0	0.2 ± 0.0*	0.5 ± 0.1	0.3 ± 0.0
20:5n-3	7.7 ± 0.6	3.7 ± 0.2*	11.8 ± 1.0	7.1 ± 0.9*	13.7 ± 1.9	9.8 ± 0.6*
22:5n-3	0.9 ± 0.0	0.4 ± 0.0*	1.2 ± 0.2	0.6 ± 0.1*	1.5 ± 0.1	1.0 ± 0.1*
22:6n-3	9.3 ± 1.1	4.5 ± 0.3*	22.5 ± 3.2	15.2 ± 3.6*	28.7 ± 3.9	23.0 ± 2.4*
Total n-3 PUFA[f]	22.2 ± 1.8	20.6 ± 0.9	38.1 ± 3.8	31.2 ± 3.1*	46.5 ± 4.7	41.8 ± 2.1
Total PUFA	27.3 ± 1.1	32.2 ± 1.1*	42.8 ± 3.9	41.7 ± 2.2	51.2 ± 4.8	52.0 ± 1.8

[a]Results are expressed as percentage of wet weight (lipid content) and percentage of total FA (FA composition) and are means ± SD ($n = 4$). Asterisks denote a significant effect of diet on the FA composition of each tissue as determined by the Student's t-test ($P < 0.05$) (41)
[b]Totals include 15:0 and 20:0 present at up to 0.3%.
[c]Includes 16:1n-9 at up to 0.2%
[d]Includes 20:1n-7 at up to 0.4%
[e]Totals include 18:3n-6, 20:2n-6 and 22:5n-6 present at up to 0.4%
[f]Totals include 20:n-3 present at up to 0.2%

these two desaturases are not known, and the cDNA have not been isolated, and so the FA specificities, tissue distributions, and hence possible functions, if any, of these desaturases are not known. The function of the third (FADS3) desaturase gene in humans is also unknown at the present time (47).

Mammalian desaturase genes have been demonstrated to be subject to nutritional regulation. The expression of Δ6 desaturase in liver was increased in mice fed triolein (18:1n-9), an EFA-deficient diet, compared with mice fed corn oil, a diet rich in 18:2n-6 (45). Similarly, the expression of both Δ6 and Δ5 desaturases was 4-fold higher in rats fed a fat-free diet or a diet containing triolein compared with that in rats fed either safflower oil (18:2n-6) or menhaden oil (n-3 HUFA) (46). Similar results have been obtained in salmonids, with dietary linseed oil (rich in 18:3n-3) increasing the expression of liver Δ6 desaturase in rainbow trout, and of liver Δ5 desaturase and elongase in Atlantic salmon, compared with levels in fish fed FO (20,25). In recent studies, expression levels of both Δ6 and Δ5 desaturases were increased in liver of salmon fed a VO blend (rich in C_{18} PUFA) compared with levels in fish fed FO, but elongase expression was not increased (22,26). In the present study, the expression of Δ6 desaturase in cod liver and intestine was not significantly increased in fish fed VO compared with that in fish fed FO, although expression of the PUFA elongase was increased in liver by dietary VO. The expression of the genes was generally reflected in the activity of the HUFA biosynthesis pathway and in the FA compositions of the tissues, which showed no evidence of any Δ6 desaturase or PUFA elongase activity, with 18:4n-3 and 20:4n-3 levels not maintained, and 18:3n-6 levels only very low and 20:3n-6 not observed at all, in fish fed VO. Previously, increased desaturase expression was reflected in higher enzyme activities in both mice (45) and salmon (25,26). In the only other study on a marine fish, expression of Δ6 desaturase in sea bream liver was higher in fish fed a HUFA-free diet compared with that in fish fed a HUFA-rich diet, but activities were not measured in that study (23).

In conclusion, the study described here has demonstrated

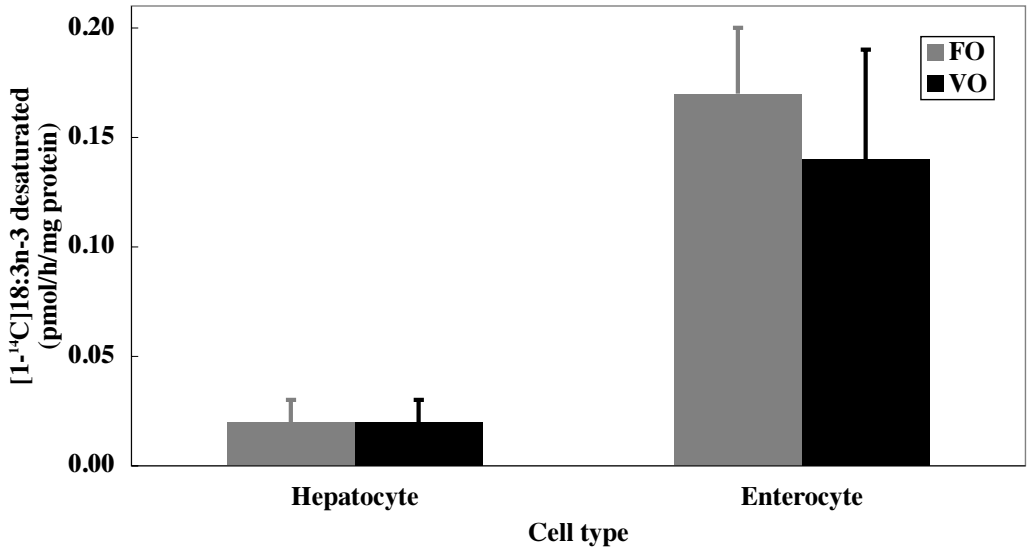


FIG. 5. Effect of diet on highly unsaturated fatty acid (HUFA) synthesis in hepatocytes and cecal enterocytes after feeding the experimental diets for 90 d. Results are means ± SD (n = 4), and represent the rate of conversion (pmol.h^{-1}.mg protein^{-1}) by desaturation/elongation of [1-^{14}C]18:3n-3 to all desaturated products. There was no significant effect of diet as determined by the student t-test (41).

that Atlantic cod express a fatty acyl desaturase gene, the product of which shows substantial Δ6 desaturase activity in a heterologous yeast expression system. The Δ6 desaturase was highly expressed in brain followed by liver, kidney, intestine, and all tissues examined. These data reinforce the impression that the poor ability of marine fish, such as cod, to synthesize HUFA is not due to lack of a Δ6 desaturase, but rather to deficiencies in other parts of the biosynthetic pathway. However, cod hepatocytes and enterocytes showed very little Δ6 desaturase or HUFA biosynthesis activity. The expression of the Δ6 desaturase and PUFA elongase genes did not appear to be under significant nutritional regulation, being generally similar

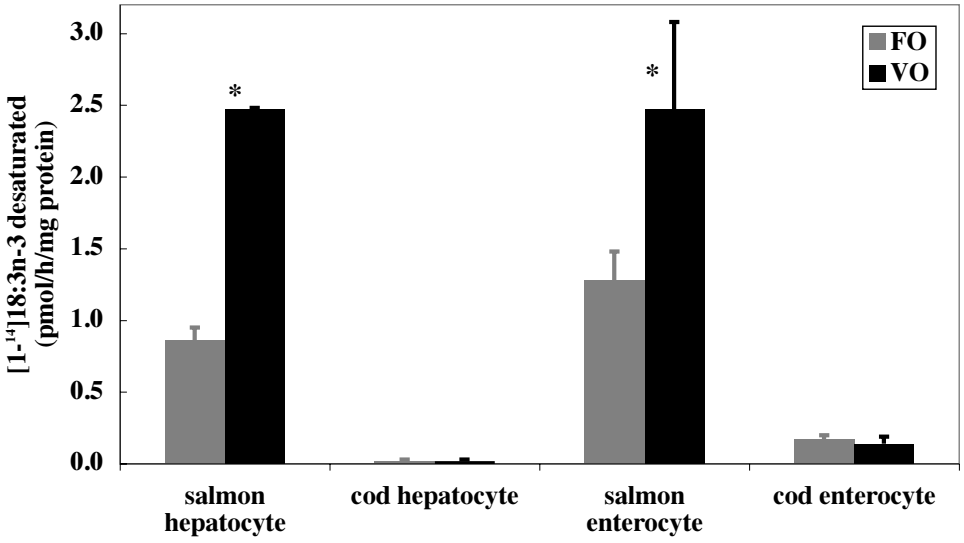


FIG. 6. Comparison of HUFA synthesis in Atlantic salmon and cod hepatocytes and enterocytes. Presentation of results is as described in the legend to Fig. 5. The salmon data were obtained in salmon smolts of similar size (220 g) to the cod in the present trial and assayed at the same time of year (July). The salmon were fed a similar FO and VO blend (26). An asterisk denotes a significant effect of diet as determined by the student t-test ($P < 0.05$) (41).

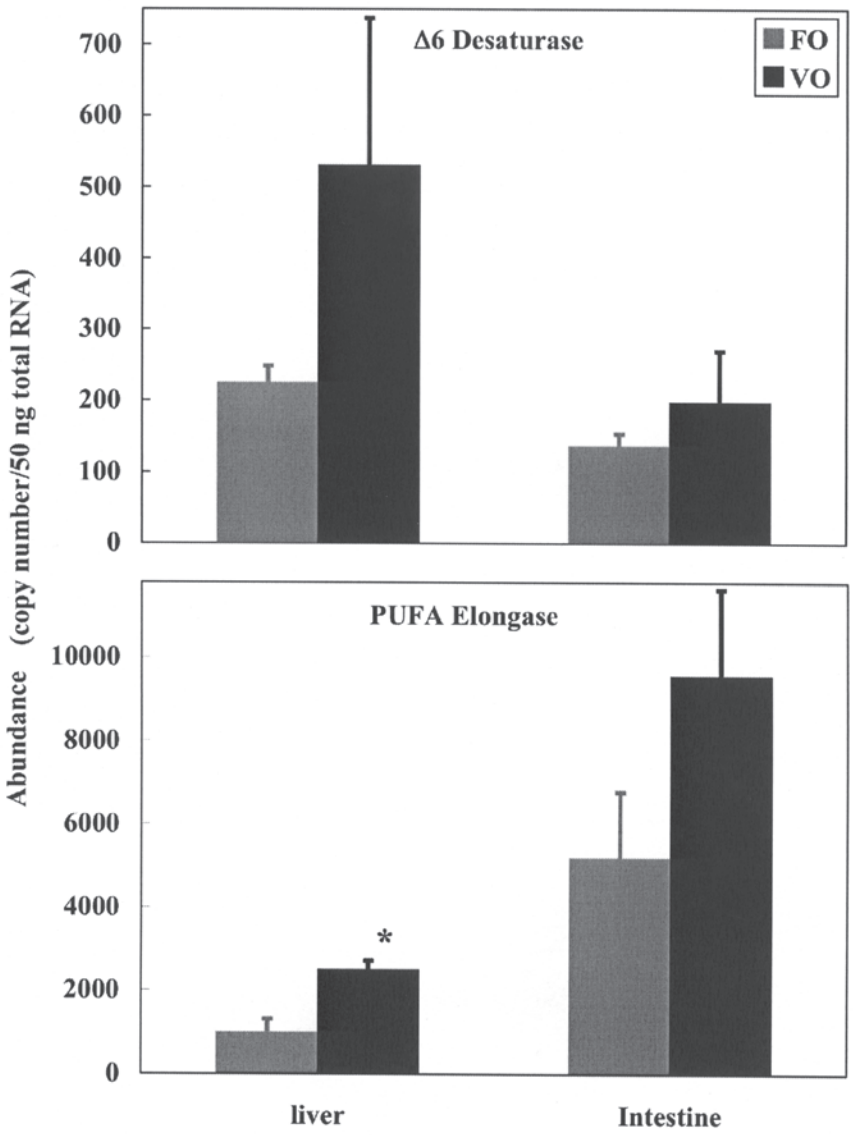


FIG. 7. Effect of dietary VOl on the expression of fatty acyl Δ6 desaturase and elongase genes in liver and intestine (pyloric ceca) from Atlantic cod. Transcript (mRNA) copy number was determined by Q-PCR and normalized as described in the text. Results are expressed as means ± SEM ($n = 4$). An asterisk denotes a significant effect of diet as determined by the student t-test ($P < 0.05$) (41).

in liver and intestine of fish fed the VO diet compared with fish fed the FO diet; this was reflected in unchanged enzyme activities in hepatocytes and enterocytes. Further studies are required to determine why the Δ6 desaturase appears to be barely functional in cod.

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Characterization of Scavenger Receptor Class B, Type I in Atlantic Salmon (*Salmo salar* L.)

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ABSTRACT: The scavenger receptor class B, type I (SR-BI) is an important player in regulation of mammalian lipid homeostasis. We therefore wanted to study this receptor in Atlantic salmon (*Salmo salar* L.), which requires a diet with particular high lipid content. We have for the first time cloned and characterized SR-BI from a salmonid fish. The predicted 494 amino acid protein contained two transmembrane domains, several putative N-glycosylation sites, and showed 72% sequence identity with the predicted homolog from zebrafish. SR-BI expression was analyzed by reverse transcription Real-Time PCR in several tissues, and a high relative expression in salmon midgut was detected, which may suggest that SR-BI has a role in uptake of lipids from the diet. We also expressed a construct of salmon *myc*-tagged SR-BI in salmon TO cells and HeLa cells, which gave a protein of approximately 80 kDa on reducing SDS-PAGE using an antibody against the *myc*-epitope. Immunofluorescence microscopy analyses of the salmon SR-BI protein in transiently transfected HeLa cells revealed staining in the cell periphery and in some intracellular membranes, but not in the nucleus, which indicated that the salmon protein may be a functional membrane protein. We also observed a high degree of co-localization using an anti-peptide SR-BI antiserum. We found that 20 μg mL^{-1} insulin up-regulated the SR-BI mRNA levels in primary cultures of salmon hepatocytes relative to untreated cells. Oleic acid, EPA, DHA, or dexamethasone did not affect the relative expression of SR-BI in this liver model system. In conclusion, the salmon SR-BI cDNA encoded a protein with several features common to those of mammalian species. SR-BI gene expression was high in the intestine, which leads us to propose that SR-BI may contribute to the uptake of lipids from the diet.

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The scavenger receptor class B, type I (SR-BI) (human homolog called CD36 and LIMPII analogous-1, CLA-1) has been characterized in a variety of mammals (1–2, reviewed in 3), non-mammalian vertebrates, and amphibians (4), and a similar receptor has even been discovered in the fruit fly (5). Mammalian SR-BI, which is a member of the CD36 receptor family, is a key player in cholesterol homeostasis (3) and was the first cell–surface, high-density lipoprotein (HDL)-receptor (6) to be molecularly well described. Glass *et al.* (7) discovered that there was a selective uptake of cholesteryl esters from HDL to rat liver, adrenals, and gonads, where there also is an abundant receptor expression closely regulated through trophic hormones. Transport of cholesterol from the liver to lipid-poor lipoprotein acceptors is also mediated through this HDL-receptor. The murine SR-BI is N-glycosylated and co-localized with caveolae (8). Two alternatively spliced transcripts of SR-BI, namely SR-BII (9) and SR-BIII (10), have been detected in mammals. They differ C-terminally, and it has been shown that at least the SR-BI and SR-BII isoforms possess various HDL-binding properties (11), while the function of SR-BIII detected in human atherosclerotic plaques has not been thoroughly studied. Mammalian SR-BI has broad ligand specificity. SR-BI is a receptor for native and modified lipoproteins (including low-density lipoprotein (LDL), oxidized LDL, acetylated LDL, HDL, oxidized HDL, very low-density lipoprotein (VLDL)), anionic phospholipids, maleylated bovine serum albumin (M-BSA), and advanced glycation end-products (AGE, as glycated BSA) (3). It has been shown that SR-BI is able to mediate phagocytosis of apoptotic cells through binding of phosphatidylserine residues on the cell surface (12). Recently, is has been shown that SR-BI also possesses other functions such as inducing ligand independent apoptosis in the absence of HDL (13) and transporting the lipophilic vitamin E across enterocytes (14). Additionally, Bietrix *et al.* (15) demonstrated a novel role of SR-BI in intestinal absorption of triacylglycesol (TAG) and cholesterol in mice.

Although various scavenger receptors have been studied in salmonids on a functional level (16-18), the SR-BI have, prior to this study, not been characterized in Atlantic salmon. Since this receptor is so important in mammalian lipid homeostasis, we wanted to clone and characterize this in Atlantic salmon, which is dependent on high-lipid diets for optimal growth (19). Hormones, fatty acids (FAs), and nutrients control the SR-BI expression levels in different tissues and species through com-

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Abbreviations: aa, amino acid; AB, antibody; BSA, bovine serum albumin; CLA-1, CD36 and LIMPII analogous-1; D, dermis; Dex, dexamethasone; DHA, docosahexaenoic acid; EF1A, elongation factor 1 alpha; EPA, eicosapentaenoic acid; FA, fatty acid; FBS, fetal bovine serum; G, gills; H, heart; HDL, high-density lipoprotein; HG, hindgut; HK, head kidney; Ins, insulin; L, liver; LDL, low-density lipoprotein; MG, midgut; nt, nucleotides; OA, oleic acid; ORF, open reading frame; PBS, phosphate buffered saline; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated FA; RACE, rapid amplification of cDNA ends; REST, relative expression software tool; S, spleen; SR-BI/II/III, scavenger receptor class B, type I/II/III; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TAG, triacylglycerol; UTR, untranslated region; VLDL, very low-density lipoprotein.

plex regulatory networks of transcription factors. Even though the SR-BI transcripts are quite well conserved through evolution, there is divergence between promoters in different species (3), and thus SR-BI's responsiveness to various ligands may be different—especially across vertebrate classes. Primary cultures of salmon hepatocytes are well suited as a model system for studies of lipid metabolism in salmon liver (20, 21). It would thus be interesting to study the expression of the receptor in a salmon primary hepatocyte culture by exposure to nutrients and hormones. Oleic acid (OA, 18:1n-9) and the n-3 (omega-3) polyunsaturated FA, (PUFAs) docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), are abundant FAs in standard fish oil and fish/meal-based salmon feed. As in mammals, EPA, but not DHA, has a reducing effect on TAG secretion in salmon hepatocytes (21); however, the mechanisms for this have not been revealed. In hamsters, PUFAs in the diet resulted in up-regulation of hepatic SR-BI mRNA and protein *in vivo* (22), whereas insulin down-regulated hepatic SR-BI (23). Atlantic salmon hepatocytes were therefore incubated with these FAs in addition to insulin and the synthetic glucocorticoid dexamethasone. Dexamethasone has been shown to up-regulate SR-BI expression in primary rat hepatocytes (24). To our knowledge, no previous studies have examined the effects of glucocorticoid hormones on SR-BI mRNA expression in fish hepatocyte cultures. However, primary salmonid hepatocytes respond to glucocorticoids in terms of many other endpoints (reviewed in 25, 26). The fish liver seems to be a main target for the glucocorticoid cortisol, coping with the increased availability of FAs through oxidation and possibly resynthesis (25). Metabolic actions of insulin in fish have also been thoroughly characterized (reviewed in 27), and studies have confirmed that salmonid hepatocyte cultures respond both to bovine and salmonid insulin (26, 28). Plasma concentrations of FAs tend to decrease after insulin administration, but knowledge about the specific effects of insulin is scarce (27). However, the regulation of lipid metabolism by insulin does not seem to follow mammalian patterns.

By conducting this study we will be able to understand more about the expression and regulation of SR-BI in Atlantic salmon. Hence, such knowledge is an important contribution to the understanding of the mechanisms through which salmonids control and regulate the high lipid levels they are dependent on for optimal growth.

MATERIALS AND METHODS

Sampling, RNA isolation, and cDNA synthesis of tissues from Atlantic salmon. Tissue samples originated from VESO Vikan (Namsos, Norway). One hundred juvenile Atlantic salmon (average size 28.2 g) were kept in one tank at 12°C under controlled conditions. Samples were harvested from the gills, livers, spleens, head kidneys, hindguts (from immediately oral to anus), midguts (from immediately aboral to pylorus and pyloric caeca), and hearts of five fish and immediately stored in RNA*later*® (Ambion Inc, TX, USA) at –20°C until RNA extraction. Total RNA was isolated using the RNeasy® Mini Kit from Qiagen (MD, USA) according to the manufacturer's instructions. DNase digestion by TURBO DNA-*free*™ reagents from Ambion was performed according to the protocol to remove potential genomic DNA contamination. cDNA was made from 0.5 μg total RNA by using random hexamer primers and reverse transcription reagents from Applied Biosystems (ABI) (CA, USA).

Isolation of hepatocytes. Atlantic salmon of approximately 500 g were kept in seawater tanks at 9°C at NIVA's Research station, Drøbak, Norway. The fish were fed a commercial diet prior to isolation of hepatocytes. The fish were anesthetized with metacain, the abdominal cavity was exposed, and the vena porta cannulated. Livers were perfused following a two-step collagenase procedure developed by Seglen (29) and modified by Dannevig and Berg (30) to isolate hepatocytes. The hepatocytes were resuspended in L-15 medium containing 2% fetal bovine serum (FBS, Gibco, Scotland), 2 mM L-glutamine, and 0.1 mg mL^{-1} gentamycin (Cambrex Bio Science). Cell viability was assessed by staining with 0.4% Trypan blue. In average, more than 95% of the cells were viable. The protein content of the cell suspension was determined according to the method described in Lowry *et al.* (31). Approximately 8×10^6 cells (approx. 12 mg protein) were plated onto 25 cm^2 flasks and left to attach overnight at 12°C.

Hepatocyte culturing, incubation with ligands, sampling, and preparations for Real-Time PCR. Hepatocytes for gene-expression studies were thoroughly washed in serum-free L-15 medium. Each flask (except the controls) was incubated with 250 μM oleic acid (OA), DHA, EPA, and dexamethasone (Dex) or 20 μg mL^{-1} (3.3 μM) bovine pancreatic insulin (Ins) in a serum-free L-15 medium. FAs, insulin, and dexamethasone were purchased from Sigma (MO, USA). The FAs were added to the medium in the form of their potassium salts, bound to bovine-serum albumin (BSA). (The molar ratio between a FA and BSA was 2.5.) The controls were incubated with phosphate-buffered saline (PBS)/BSA (100 μM) only. There were six parallels for each treatment. After incubation for 48 h at 12°C, the cells were harvested and kept at –80°C until RNA extraction. Total RNA was isolated from hepatocytes, and cDNA was made as described above for the tissue samples from 2 μg total RNA. Analyses were duplicated in the reverse transcription step.

Production of a polyclonal antibody against salmonid SR-BI. Before cloning and sequencing full-length salmon SR-BI, we wanted to make a polyclonal AB against the protein based on a partial sequence from rainbow trout (*Oncorhynchus mykiss*). The partial SR-BI sequence from rainbow trout (GenBank accession no. DQ266043) obtained in our lab was translated and aligned with SR-BI amino acid (aa) sequences from other species. (See Table 2 in the Results section for GenBank accession numbers.) The peptide (FEPSMSVGNESDV) that was selected is situated in the extracellular loop of SR-BI and was coupled to keyhole limpet hemocyanin (KLH) carrier. Polyclonal antibody was produced by Eurogentec S.A. (Belgium) by immunization of two rabbits, followed by affinity purification against the peptide.

TABLE 1
Primers for RACE, SR-BI ORF Cloning and Quantitative Real-Time PCR

Target	Direction	Sequence (5′-3′)	Amplicon size (bp)	GenBank acc. no.
GSP 1[a]	Antisense	GGAAGTCCACCAGCTTGCTGTCGTAG	–	DQ266043
GSP 2[a]	Sense	AGCAGAACATCACGTTCCATCCCAAC	–	DQ266043
5′ UTR[a]	Sense	ACACTCGACTTCAAGACAAGCCTTA	–	DQ914655
3′ UTR[a]	Antisense	TGGGGTTCCAGTGATCATCAGTCTC	–	DQ914655
SR-BI ORF[b]	Sense	GGGG*GAATTC*ACCATGAATAAATCTAAATTAGCGATC	1500	DQ914655
	Antisense	GGGG*AAGCTT*GTCCACAGGGTCCTGTATTAGAG		
SR-BI[c]	Sense	AACTCAGTGAAGAGGCCAAACTTG	204	DQ914655
	Antisense	TGCGGCGGTGATGATG		
EF1A[c]	Sense	CACCACCGGCCATCTGATCTACAA	77	AF321836
	Antisense	TCAGCAGCCTCCTTCTCGAACTTC		
18S rRNA[c]	Sense	TGTGCCGCTAGAGGTGAAATT	61	AJ427629
	Antisense	GCAAATGCTTTCGCTTTCG		

[a]Primers for RACE.
[b]Primers for cloning of SR-BI ORF; restriction sites in italics (EcoRI or HindIII), and start codon (ATG) underlined.
[c]Primers for quantitative Real-Time PCR.

Sequence information and primer design. Primers for random amplification of cDNA ends (RACE) and Real-Time PCR were designed by use of Primer3 (32) and PrimerExpress 2.0 (ABI), respectively (Table 1). Primers for subsequent steps in the cloning procedure were designed manually (Table 1).

Cloning of salmon SR-BI by RACE. Cultured hepatocytes were harvested, and total RNA was isolated and treated with DNase as described above for the tissue samples. SMART™ RACE cDNA amplification kit (BD Biosciences Clontech, CA, USA) and 1 μg total RNA were used to make 5'- and 3'-RACE-Ready cDNA. The RACE was performed using the gene-specific primer (GSP) 1 and GSP 2 (Table 1) for the 5' and 3' reactions, respectively, combined with the primers in the kit. A touchdown PCR was set up as follows: 5 cycles of 94°C 30 s, 72°C 3 min; 5 cycles of 94°C 30 s, 70°C 30 s, 72°C 3 min; and 25 cycles of 94°C 30 s, 68°C 30 s, 72°C 3 min. The 3' RACE was run for an additional 10 cycles of the latter parameters to obtain detectable products. PCR products were then TA subcloned into TOPO® Vectors (Invitrogen, CA, USA) and sequenced (GATC Biotech, Germany). After sequence analyses, new primers were designed in 5' untranslated region (UTR) and 3' UTR (Table 1), based on the obtained salmon sequence. Full-length cDNA was amplified using the 5' RACE cDNA as a template and Advantage 2 PCR reagents (BD Biosciences Clontech) in 50 μL volumes. The following PCR program was run: 94°C 1 min; 35 cycles of 94°C 30 s, 58°C 45 s, 68°C 2 min; and a final extension of 70°C 10 min. PCR products were subcloned and sequenced as described above.

Cloning of the SR-BI open reading frame (ORF) into an expression vector. A PCR with primers (Table 1) defining SR-BI ORF and including restriction sites was run at 95°C 2 min; 30 cycles of 95°C 30 s, 58°C 30 s, 68°C 2 min; and a final extension of 72°C 10 min. pcDNA™3.1/*myc*-His(-) A and the purified PCR product were double digested with EcoRI and HindIII restriction enzymes from New England Biolabs (NEB, MA, USA). The vector was treated with CIAP (Fermentas Inc, MD, USA), and a ligation was performed using T4 DNA ligase (Invitrogen) according to the manufacturer's instructions. Verification of correct insertion was done by sequencing. The construct is denoted pSR-BI-*myc*-His.

Transfection of human HeLa and salmon TO cells. HeLa cells were grown in Dulbecco-modified Eagle-Earle medium (D-MEM) (Gibco) containing 10% vol/vol FBS, 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate (Biochrom

TABLE 2
Percentage of Identities between the SR-BI Amino Acid Sequences of Different Species and Atlantic Salmon Deduced Amino Acid Sequence

Species	GenBank accession no.[a]	Identity (%)	Expected value
Human	NP_005496	58	$3e^{-158}$
Pig	NP_999132	56	$2e^{-155}$
Cow	AAB70920	55	$1e^{-152}$
Mouse	Q61009	54	$3e^{-149}$
Rat	P97943	55	$1e^{-150}$
Zebrafish	NP_944603	72	0.0

The BLAST 2 SEQUENCES program was used for pair-wise protein-protein sequence comparison (Blastp 2.2.14).
[a] The same as in Fig. 2.

AG, Germany), and penicillin-streptomycin (10,000 units of penicillin mL^{-1} and 10,000 µg of streptomycin mL^{-1}) (Gibco), at 37°C in a 5%-CO_2 incubator. The cells were plated at 5×10^5 cells in 21.5-cm^2 dishes with glass coverslips the day before transfection. They were transfected with 1 µg DNA (pSR-BI-*myc*-His) using FuGENE® 6 (Roche Applied Science, IN, USA) according to the manufacturer's instructions. The cells were then cultured for 48 h.

1.5 million salmon TO cells (head kidney cell line, passage 50) (33) were transfected using 5 µg DNA (pSR-BI-*myc*-His) and the Nucleofector™ technology from Amaxa Biosystems (MD, USA) and seeded in six-well culture dishes. The TO cells were cultured at 20°C in an L-15 medium containing 5% FBS, 2 mM L-glutamine, and 0.1 mg mL^{-1} gentamycin for 72 h.

Both cell lines were also transfected with pEGFP-N1 (Clontech Lab., CA, USA) as positive controls for transfection efficiency.

Immunofluorescence analysis. Transfected and untransfected HeLa cells on coverslips were washed in cold PBS and fixed with 4% paraformaldehyde in PBS (Electron Microscopy Sciences, PA, USA) for 20 min, then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 5 min, and washed with PBS. Unspecific staining was blocked by incubating the cells with 2% FBS in PBS for 30 min. The cells were incubated with one or both primary antibodies (ABs)—mouse anti-*myc* (kindly provided by Professor Harald Stenmark, Dept. of Biochemistry at the Norwegian Radium Hospital, Norway) diluted 1:50 in 2% FBS in PBS and anti-peptide SR-BI undiluted—for 60 min. After three washes in PBS, the cells were labeled for 60 min with secondary ABs conjugated to CY-2 and/or CY-3 (Jackson ImmunoResearch Laboratories Inc, PA, USA). The coverslips were washed three times in PBS and mounted in ProLong Gold (Molecular probes, OR, USA). Cells were then examined with a Nikon C-1 confocal mounted on a TE 2000E inverted microscope.

DNA and amino-acid sequence analysis. All nucleotide sequences obtained were assembled in ContigExpress in Vector NTI advance 9.1.0. (Invitrogen), and a consensus sequence was obtained. TBLASTX searches were performed to identify the protein. TMHMM 2.0 (34) was used to search for transmembrane domains and NetNGlyc 1.0 (35) was used to find potential N-glycosylation sites. Moreover, Simple Modular Architecture Tool (SMART) analyses (36) were performed to reveal other possible domains. The BLAST 2 SEQUENCES (bl2seq) program was used for pair-wise protein-protein sequence comparison (Blastp 2.2.14) (37). CLUSTALW (38) was used for multiple sequence analyses.

Quantitative Real-Time PCR analyses. Real-Time PCR was performed with the ABI Prism® 7000 system and gene-specific primers (Table 1). A 2× SYBR® Green PCR Mastermix (ABI), 0.4 µM of each primer, and the cDNA template (both from tissue samples and hepatocytes) were mixed in 25 µL volumes. Standard curves for each primer pair were calculated by serial dilution, and PCR efficiencies (E) were calculated according to the formula $E = 10^{(-1/slope)}$ (39). A two-step PCR was run for 40 cycles (15 s at 95°C, 1 minute at 60°C) with an initial incubation of 2 min at 50°C and 10 min at 95°C to activate the hot-start AmpliTaq Gold® DNA Polymerase. Specificity of the PCR products was verified by agarose-gel electrophoresis and amplicon sequencing.

Data analyses and statistics. Relative quantities of SR-BI transcription levels in the tissue samples were calculated by the $2^{-\Delta\Delta Ct}$ method (40), adjusted for PCR efficiency. Elongation factor 1 alpha (EF1A) was used as a reference gene to normalize data in these analyses (41). The mean values of all the tissues were related to the tissue sample with the lowest ΔCt value (hindgut).

Relative expression of SR-BI in hepatocytes was determined using the Relative Expression Software Tool (REST-384© – version 1) (42). A comparison of gene expression in hepatocytes cultured in normal conditions versus hepatocytes treated with different FAs and synthetic ligands were performed and normalized to the two reference genes 18S rRNA and EF1A (41). This analysis sets expression level (ratio between SR-BI and reference genes) in the untreated hepatocytes to 1 and the changes in the test groups (OA, EPA, DHA, Ins, and Dex) relative to this. Significant difference from expression in control hepatocytes was calculated by the Pair Wise Fixed Reallocation Randomization Test© (2000 randomizations) in the software. All samples were reverse transcribed and amplified in parallel, and mean values were used in REST, n = 6. Probability values (*P*) of less than 0.05 were considered significant.

Western blot analysis of transfected HeLa and TO cells. Transfected HeLa and TO cells were harvested in PBS, spun down, and resuspended in Laemmli sample buffer (containing β-mercaptoethanol) for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and subsequent Western analysis. Cell lysates were loaded and separated on a 10%-SDS polyacrylamide gel. Proteins were then electroblotted onto a methanol-activated Hybond™-P membrane (Amersham Biosciences). The membrane was pre-incubated in a blocking solution containing 5% skimmed milk in PBS/0.1% Tween 20, which was the basis for all subsequent dilutions of ABs. The blot was then incubated with a mouse anti-*myc* (1:200) primary AB, followed by goat anti-mouse HRP-IgG AB (1:5000) (Jackson ImmunoResearch Laboratories Inc) and chemiluminescent detection. A loading control was introduced by re-hybridizing the blot with a rabbit anti-actin (1:500) AB (Sigma, Product no. A2066) and a goat anti-rabbit HRP-IgG AB (1:10000) (Jackson ImmunoResearch Laboratories Inc).

RESULTS

Sequence analysis of salmon SR-BI cDNA. To obtain the salmon SR-BI cDNA, RACE was performed on salmon hepatocyte RNA using primers based on a partial sequence from rainbow trout. The resulting cDNA sequence of 1852 nucleotides (nt) contained a 280 nt 5' untranslated region (UTR), an open reading frame (ORF) of 1482 nt, and a 90 nt 3' UTR (Fig. 1; GenBank accession no. DQ914655). Translation of the ORF gave a putative protein of 494 aa. Further analyses of the

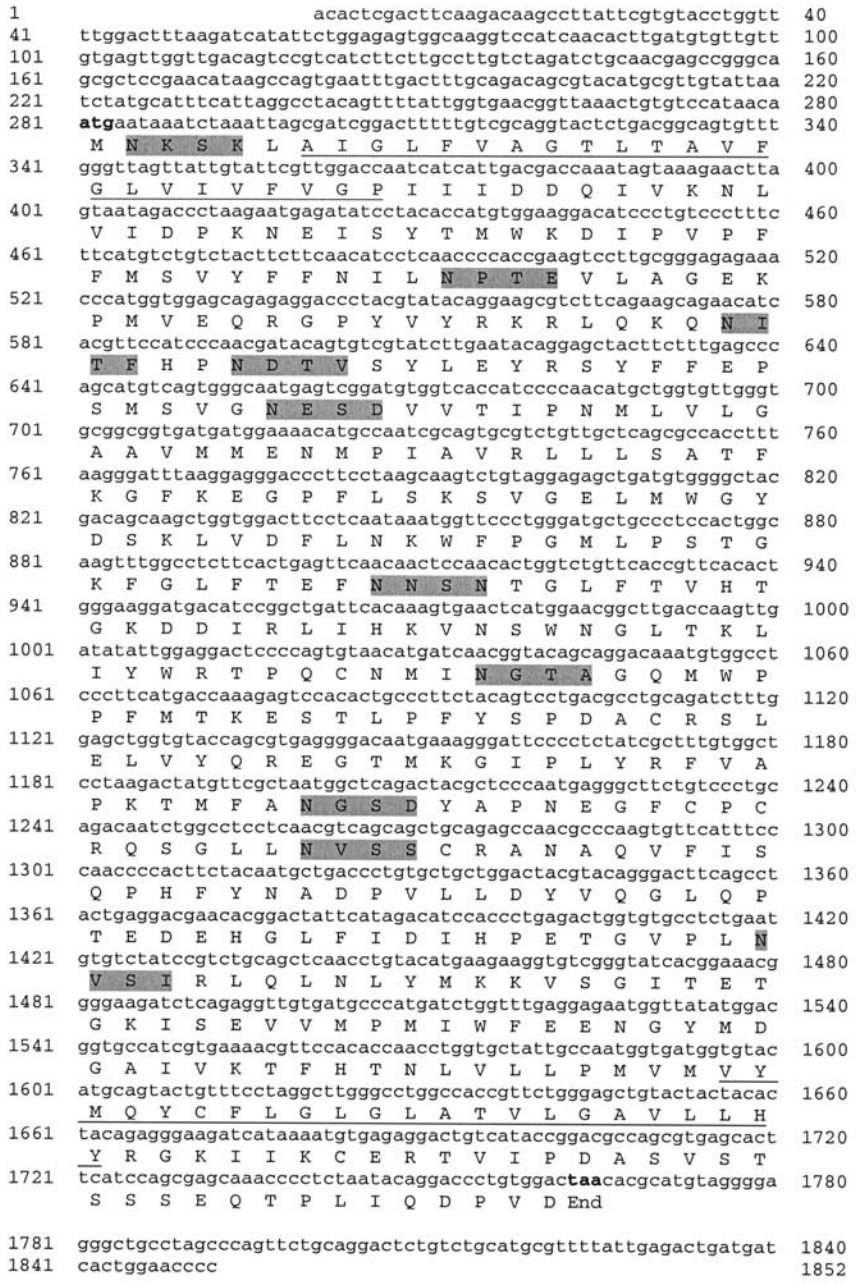

FIG. 1. Salmon SR-BI cDNA nucleotide sequence and its predicted amino acid sequence. Putative translation start codon (atg) and stop codon (taa) are indicated in bold. The positions of the N-terminal and C-terminal transmembrane domains are underlined, and the positions of the putative N-glycosylation sites are indicated with grey boxes. (GenBank accession no. DQ914655.)

predicted protein revealed two primary putative transmembrane alphahelical domains of 23 aa each (residues 7-29 and 439-461) and ten putative N-glycosylation sites (residues 2, 71, 99, 105, 126, 209, 252, 307, 327, and 380). A CD36 domain including aa 1 to 442 (E-value = 3.8e-180) was also discovered.

The deduced protein sequence shared overall similarity to SR-BI of other species (Fig. 2). All species contain the same phylogenetically conserved CXXS redox and GXXXG motifs. The leucine residues in a seven-heptad repeat-putative leucine zipper are not conserved between mammals and fish. Sequence identity obtained from BLAST 2 SEQUENCES analyses are shown in Table 2. Salmon SR-BI showed the highest identity to zebrafish (72%), and the lowest to mouse (54%), SR-BI.

Expression of SR-BI mRNA in salmon tissues. Relative expression of SR-BI mRNA in a selection of salmon tissues was investigated by reverse transcription Real-Time PCR and analyzed by the $2^{-\Delta\Delta Ct}$ method. Particular high expression was dis-

FIG. 2. Comparison of the predicted salmon SR-BI protein sequence with pig, cow, human, mouse, rat, and zebrafish SR-BI homologs. The sequences were aligned in CLUSTALW. "*" below alignment blocks denotes residue identities, ":" denotes conserved substitutions, and "." denotes semi-conserved substitutions. The 13 aa-peptide used for AB production is indicated in bold at residue 118-130, relative to the start of salmon SR-BI. The black box in the alignment denotes a conserved CXXS redox motif, and the grey box denotes a conserved GXXXG motif. Small black boxes below the alignment denote the leucine (L) residues in a seven-heptad repeat-putative leucine zipper proposed in mammals. Accession numbers are listed in Table 2.

covered in midgut (from immediately aboral to pylorus and pyloric caeca) (Fig. 3)—approximately 120-fold more than in the tissue with lowest expression, namely hindgut (from immediately oral to anus). Comparatively little mRNA was present in all other tissues investigated.

Transfection studies with the salmon pSR-BI-myc-His fusion construct. To further characterize the salmon SR-BI we constructed an expression vector with the putative ORF of salmon SR-BI in frame with a *myc*-His tag. We wanted to investigate the cellular localization in transiently transfected cells and hence used HeLa cells as a model system, as these cells are easy to work with and have high transfection efficiency (approximately 80–90%). Transfected HeLa cells were double labeled with the anti-peptide polyclonal SR-BI AB and an anti-

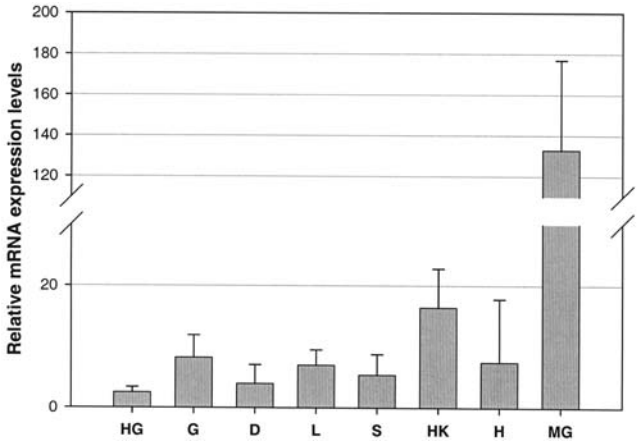

FIG. 3. Relative expression of SR-BI mRNA in salmon tissues; $2^{-\Delta\Delta Ct}$ analysis of the mRNA expression of SR-BI relative to mRNA levels of EF1A in eight salmon tissues. Data was normalized to the lowest relative expression (one HG sample). HG = hindgut (from immediately oral to anus), G = gills, D = dermis, L = liver, S = spleen, HK = head kidney, H = heart, and MG = midgut (from immediately aboral to pylorus and pyloric caeca). Data are means with standard deviation error bars. N = 5 for HG, G, D, L, and HK. N = 4 for S and MG.

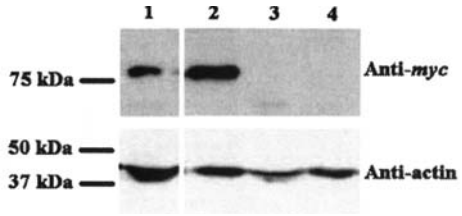

FIG. 5. Western blot analysis of the SR-BI-*myc*-His fusion protein in transiently transfected TO and HeLa cells detected with anti-*myc* AB. Transfected salmon TO (lane 1) and HeLa cells (lane 2) produce a protein of about 80 kDa. There were no bands in the untransfected controls for HeLa (lane 3) and TO (lane 4) cells. An anti-actin antibody was used as a loading control.

myc AB. Both antibodies stained the cell at the periphery and in the intracellular membranes, but not in the nucleus (Fig. 4).

Furthermore, to verify that the extent of glycosylation in salmon and human cells was comparable we transfected both HeLa cells and salmon TO cells with the pSR-BI-*myc*-His construct for Western analysis. A protein of about 80 kDa was detected for both cell types on a Western blot (reducing conditions) using an anti-*myc* AB (Fig. 5). We were not able to detect the fusion protein with the polyclonal AB made against salmonid SR-BI (data not shown).

Regulation of SR-BI gene expression in salmon hepatocytes. SR-BI expression in mammals is regulated in response to FAs and other ligands, and we investigated this in salmon-primary cultures of hepatocytes. We focused on some FAs relevant to salmon physiology (OA, EPA, and DHA), but also insulin and dexamethasone, which have been widely tested in mammalian assays. REST analyses of Real-Time PCR data showed that there was a 2-fold increase in the relative expression level of SR-BI in hepatocytes treated with 20 μg mL^{-1} insulin (Fig. 6).

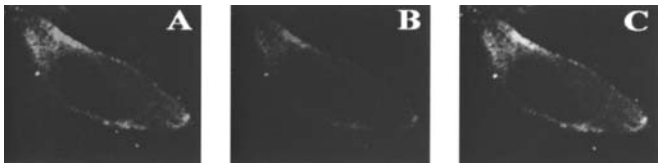

FIG. 4. HeLa cells transiently transfected with the pSR-BI-*myc*-His construct and visualized by confocal immunofluorescence microscopy. The cells were double-labeled with anti-peptide SR-BI/CY2-conjugated mouse anti-rabbit IgG (panel A) and anti-*myc*/CY3-conjugated rabbit anti-mouse IgG (panel B). An overlay is presented in panel C. No staining was detected with secondary ABs only (data not shown).

We did not detect any changes in the SR-BI expression level in hepatocytes treated with OA, EPA, DHA, or dexamethasone.

DISCUSSION

The coding region of SR-BI in salmon had 72% aa identity with zebrafish and between 54–58% aa identity with the SR-BI from other mammalian species. Transfection studies in both mammalian and salmon cells using a *myc*-tagged salmon SR-BI, gave an approximately 80 kDa protein by SDS-PAGE and immunodetection of the *myc*-epitope. In other species, the molecular weight of SR-BI varies slightly from 84–86 kDa in frog and gold fish (4) to 82 kDa in mammals (8). There are clearly domains in the receptor that are well conserved across vertebrate species (see Fig. 2), but there are also differences, partic-

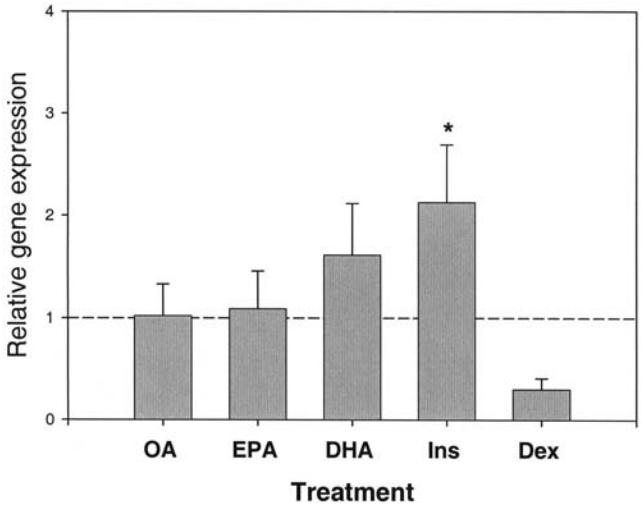

FIG. 6. Relative expression of salmon SR-BI mRNA in hepatocytes treated with OA, EPA, DHA, insulin (Ins), and dexamethasone (Dex). Dashed line denotes the ratio of SR-BI, and the two reference genes (18S rRNA and EF1A) in untreated hepatocytes (control set to 1). Significant difference from expression in control cells was calculated by the Pair Wise Fixed Reallocation Randomization Test© (2000 randomizations) in the REST©-384 software and is denoted with an asterisk (*), n = 6, $P < 0.05$.

ularly between mammals and fish in the N- and C-terminal regions.

One particularly interesting domain is the highly conserved CXXS redox motif that is present in all species investigated. In mammals, this motif has been suggested to be involved in a novel ligand-independent apoptotic pathway induced by SR-BI through activation of the caspase-8 pathway and regulated by endothelial nitric-oxide synthase (eNOS) and HDL (13). The authors hypothesized that SR-BI may play a pivotal role in the development of atherosclerosis when HDL levels are low and oxidative stress causes the relocation of eNOS away from caveolae. Furthermore, they proposed that SR-BI will contribute to the clearance of apoptotic cells to prevent further inflammatory damage to neighboring cells. It would be interesting to investigate whether the conserved CXXS motifs have similar functions in salmonids that have high levels of HDL (43) but at the same time have a high prevalence of coronary atherosclerotic lesions (reviewed in 44). However, the formation of atherosclerotic lesions in salmonids is probably caused by a combination of vascular injury, high growth rates, or stress associated with spawning migrations.

Another conserved motif near the C-terminal transmembrane domain is GXXXG, which is critical for proper delivery and metabolism of HDL-CE (45). The leucine residues in a seven-heptad repeat-putative leucine zipper in the same area (46) have been proposed to bind HDL via the amphipathic α-helical repeat units in apolipoprotein AI (47, 48). But most of these leucine residues are only semi-conserved in salmon and zebrafish. However, SR-BI is known to bind several ligands, and not much is known about the precise domains responsible for this interaction.

There are some conserved cysteine residues in the SR-BI across species, and thus these proteins have the potential to form extracellular disulfide bridges. There are also many potential N-glycosylation sites for the salmon protein as for mammalian SR-BI (8, 46). In summary, there are many structural similarities between salmon and mammalian SR-BI, and hence it is likely that SR-BI is also functionally conserved phylogenetically.

There was a 100% aa match in sequence between the partially deduced rainbow trout and Atlantic salmon SR-BI in the region that we chose for AB production. The polyclonal AB did not work in Western analyses, but we obtained good co-localization with the *myc*-epitope in transfected HeLa cells. This may suggest that our anti-SR-BI serum only recognizes the native form of the protein.

In this report we found a particularly high SR-BI mRNA expression in midgut. It is not known how closely SR-BI mRNA expression reflects SR-BI protein levels in fish (due to lack of good antibodies), but in mammalian tissues and cell systems mRNA and protein levels of SR-BI correlate well in several reports (6, 22, 49, 50). Atlantic salmon requires a high lipid content in the feed (19), and since the pyloric caeca and the proximal intestine are the main sites of fat digestion and absorption (51, 52) we propose that salmon SR-BI may be involved in lipid absorption in this part of the intestine. In mammals, on the other hand, it has been reported that the SR-BI has the highest expression in tissues with significant roles in cholesterol metabolism, namely the liver and steroidogenic tissues, but also, in lesser amounts, in tissues with critical functions in transport and lipid metabolism, including the heart and the intestine (6, 53). The receptor primarily has a function in selective uptake of CE from lipid-rich lipoproteins to the liver (reverse cholesterol transport) and secretion of excess cholesterol through the bile or efflux, and recycling of free cholesterol to lipoprotein acceptors. A recent study of mice over-expressing intestinal SR-BI has discovered a functional role *in vivo* for SR-BI in cholesterol and TAG intestinal absorption (15). Lately SR-BI has also been shown to be involved in vitamin E transport across mammalian enterocytes (14). Kiefer *et al.* (5) found that the *ninaD* gene encodes a class B scavenger receptor, which mediates uptake of dietary carotenoids, thus making them available for subsequent cell metabolism. However, there are only 26- and 27% aa identities between human and salmon SR-BI and the putative NinaD protein, respectively. In addition, the conserved CXXS and GXXXG motifs that are present in vertebrate SR-BI are not present in the NinaD protein. It would therefore be of interest in the future to further investigate the expression of SR-BI in salmon enterocytes and its potential role in the uptake of fat-soluble vitamins. Thus, evidently new functions of the SR-BI are currently discovered, and because of the highly variable physiology of mammals and lower vertebrates, particularly in terms of lipid plasma profiles and feed requirements, one can also suspect that salmon SR-BI has an important function in intestinal lipid absorption.

Salmon hepatocyte cultures have been used for two decades (54) as a model system to study various aspects of liver biology. Using primary cultures is generally advantageous compared to using cell lines, which have a tendency to become morphologically and biochemically changed over time. Treatment of salmon hepatocyte cultures with insulin gave a two-fold increase in expression levels of SR-BI (using two reference genes for normalization) relative to untreated cells. How gene transcription responds to external stimuli is largely determined by the promoter and other regulatory sequences surrounding the coding region. Promoters of the rat and human SR-BI genes have been characterized and include a number of essential elements to explain its regulation by cellular cholesterol levels and hormones (3). Even though the promoters of rat and man have many common elements, there are differences in numbers and placement, and some unique motifs exist in either one. We do not know if the salmon SR-BI promoters share the same regulatory elements as in rat and man, but promoters of other salmonid genes have shown that several common elements are conserved (55–57).

Metabolic responses to insulin occur at similar concentrations in salmonid hepatocyte culture to the expected range in hepatic portal insulin levels ($0.5 \times 10\text{-}9\text{–}15 \times 10^{-9}$ M) (27), and these cells have been shown to respond both to bovine and salmonid insulin (26, 28, 58). The stimulation of SR-BI mRNA expression we observed here occurred at a level above the physiological concentration of insulin. However, the interac-

tion between bovine insulin and the salmon insulin receptors may be weaker (reviewed in 59), and, therefore, higher concentrations may be required for activation of the receptor. Insulin has been shown to have an impact on lipid metabolism in mammalian hepatocyte cultures by influencing the secretion of several apolipoproteins (60-62). Hepatic SR-BI was reduced in hamsters after chronic infusions with insulin (23). In contrast, one study found that insulin increased the expression of SR-BI mRNA in rat theca-interstitial cells under both *in vivo* and *in vitro* conditions (63). Insulin has been shown to increase PPARγ2 expression in cultured mouse hepatocytes (64), and SR-BI expression increased in various cell types, including primary rat hepatocytes, in response to PPARγ agonists (65). However, we do not know anything about the underlying mechanisms for the increase in SR-BI expression in salmon hepatocytes observed here.

In the present study, there seemed to be a reduction in SR-BI mRNA levels for the hepatocytes treated with dexamethasone, but this was not significant, even though this synthetic glucocorticoid binds to the hepatic glucocorticoid receptor with high affinity in salmonids (25). Salmon hepatocytes were extremely sensitive (10^{-12} M and above) to dexamethasone in terms of inhibiting the response to growth hormone (26). In primary rat hepatocytes dexamethasone increased the expression of SR-BI and ABC transporter A1 (ABCA1), while it decreased the expression of both genes in a human hepatoma cell line (HepG2) (24). Effects of dexamethasone are both species and concentration dependent, and the range of dexamethasone we used here was much higher (250 μM) than in the studies mentioned above. Furthermore, we did not observe any regulation in SR-BI expression in response to either oleic acid or the PUFAs EPA and DHA, while in hamsters hepatic SR-BI mRNA and protein were enhanced in response to PUFAs (22).

In summary, there are many differences in regulation of SR-BI between species. So, even though the deduced salmon SR-BI protein had high similarity with SR-BI of other species and was expressed as a membrane protein in transfected cells, we cannot conclude that our clone is a functionally conserved HDL receptor. The high level of this transcript in the gut makes it tempting to propose that the SR-BI in salmon has an important function in the uptake of lipids from the intestine.

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Modification of Fillet Composition and Evidence of Differential Fatty Acid Turnover in Sunshine Bass *Morone chrysops* × *M. saxatilis* Following Change in Dietary Lipid Source

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ABSTRACT: Marine oil-based finishing diets have been used to restore fillet FA profile in several "medium-fat" fleshed aquaculture species, and a simple dilution model describing FA turnover has been established to predict and tailor final fillet composition. We evaluated finishing diet efficacy and suitability of the dilution model to describe patterns of FA change in a lean-fleshed model, sunshine bass. Two practical diets (45% crude protein, 15% crude lipid) were formulated, respectively containing corn oil (CO) or menhaden oil (MO) as the primary lipid sources. Sunshine bass (age 1 [~14 mo], 347 ± 8.6 g, mean individual weight ± SEM) were stocked in a recirculating system and fed the diets according to different feeding regimens during the final 28 wk of the production cycle. Control groups were fed the CO or the MO feeds exclusively; whereas, the remaining treatment groups were transitioned from the CO diet to the MO diet at 4-, 8-, or 12-wk intervals. Upon completion of the feeding trial, fish were harvested, and production performance and fillet composition were assessed. Replacing MO with CO as the primary lipid source in sunshine bass diets yielded fillets with distinctly different FA profiles; however, finishing with a MO-based diet offered significant compensation for CO-associated reductions in fillet long-chain highly unsaturated FA (LC-HUFA). Although complete restoration was not observed, we achieved significant augmentation of endogenous n-3 FA within 4 wk of feeding the MO diet, and observed a significant increase in LC-HUFA and a beneficial shift in n-3:n-6 FA ratio after 8 weeks. Simple dilution accurately predicted tissue composition for most FA; however, deviations from the model were noted, suggesting selective retention of n-3, PUFA, and LC-HUFA and preferential catabolism of saturates. We conclude marine oil-based finishing diets can rapidly augment beneficial FA levels in sunshine bass fillets; however, simple dilution models do not fully describe selective FA metabolism observed for this lean-fleshed fish.

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Abbreviations: CO, corn oil; FCR, food conversion ratio; HSI, hepatosomatic index; IP, intraperitoneal; LC-HUFA, long-chain highly unsaturated FA; MO, menhaden oil.

Marine oils derived from feed-grade, reduction fisheries often serve as the primary lipid constituents of aquaculture diets because of their high palatability to cultured animals, attractant properties, and historically widespread availability and competitive pricing. Unfortunately, exhaustive harvests and declining productivity of reduction fisheries (1) have caused marine oil prices to increase, along with pressure from conservationists to reduce use of marine animal-derived products (2). In addition to cost and availability issues, marine oils have recently been implicated as vectors of metals and various organic contaminants found in farm-raised fish (3), precipitating immense scrutiny of aquaculture from the scientific and lay communities. Although the validity of these concerns may be individually questioned (4,5), all professionals within fisheries and aquaculture recognize their cumulative impact and the limitations of capture fisheries in keeping pace with the growing demands of aquafeed production (5,6).

Sustainable expansion of aquaculture requires use of alternative lipid sources, but modified feed formulations may alter the final FA composition of cultured products and limit their nutritional value to human consumers. Marine oils contain substantial amounts of long-chain highly unsaturated FA (LC-HUFA, carbon chain length ≥20 and double bonds ≥3) that impart benefits beyond basic nutritive value (7,8) and have been shown to modulate a range of human health conditions (9,10,11,12). Tissue FA composition of fishes largely reflects the diet (13,14), and thus fillets of fish fed marine oil-based diets contain substantial amounts of bioactive LC-HUFA such as arachidonic (20:4n-6), eicosapentaenoic (20:5n-3), and docosahexaenoic (22:6n-3) acids. Conversely, oils derived from terrestrial plants do not contain these compounds, and reductions in LC-HUFA have been demonstrated following their incorporation in diets for sunshine bass *Morone chrysops* × *M. saxatilis* (15), rainbow trout *Oncorhynchus mykiss* (16), Atlantic salmon *Salmo salar* (17), gilthead seabream *Sparus aurata* (18), and red seabream *Pagrus auratus* (19). The functional nutritional value of these modified products is thereby reduced (20), linking alternative lipids with reductions in the nutritional benefit of consuming fillets from cultured fish.

To mitigate conflicting demands of sustainability and product value, aquaculture nutrition research groups have evaluated implementation of "finishing diets" at the end of the produc-

tion cycle to restore LC-HUFA content to fillets of several aquaculture species (6). Partial restoration of fillet LC-HUFA content was achieved using finishing diets in "medium-fat" species (approximately 2–8% lipid in fillet, fresh-weight basis [21]) such as turbot *Psetta maxima* (22) and Atlantic salmon (23–25). A simple dilution model (Fig. 1) has been used to describe FA turnover following dietary modification in these species (14,26–28), and has been suggested as a means of gauging feed management and finishing strategies to tailor final FA composition of cultured products. Although alternative lipid sources have been widely researched in many aquaculture species, finishing diets and FA turnover have not previously been evaluated using a lean-fleshed model (generally <2% [21]). Sunshine bass are interspecific hybrids of female white bass *Morone chrysops* and male striped bass *M. saxatilis* (reciprocal cross hybrid striped bass) and are widely cultured in freshwater systems for their mild-flavored, lean flesh. Accordingly, our objectives were to evaluate production performance and fillet composition of sunshine bass fed a low LC-HUFA content (plant-derived lipid) diet, tissue FA composition and turnover following implementation of a high-LC-HUFA content (marine-derived lipid) finishing diet, and the suitability of the established dilution model to describe patterns of FA change in this fish.

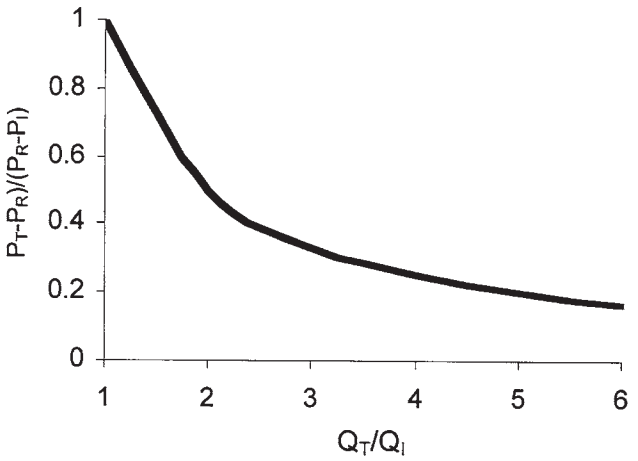

FIG. 1. Theoretical model of FA dilution within fish tissues following dietary change, where $(P_T - P_R)/(P_R - P_I)$ represents the ratio of relative change in fillet FA and Q_T/Q_I represents the relative change in total lipid (14).

EXPERIMENTAL PROCEDURES

Diet preparation and analyses. We formulated two isocaloric, isonitrogenous practical diets to contain 45% crude protein and 15% crude lipid (Table 1) and had them manufactured (extrusion processed, floating pellets) by a commercial aquafeed producer. Diets were formulated to meet all known nutritional requirements of growing sunshine bass (29) and differed only in lipid source. The formulations were designed to be generally characteristic of diets used in sunshine bass culture, but represent a plant-derived lipid "production diet" and a marine animal-derived lipid "finishing diet" to be evaluated during grow-out and at the end of the production cycle, respectively. The production diet utilized corn oil (CO) as the predominant lipid source, whereas the finishing diet contained menhaden (*Brevoortia* spp.) oil (MO), thus generating two distinct dietary FA profiles (Table 2). Although other plant-derived products such as canola and soybean oils possess greater 18-carbon n-3 FA content than corn oil, they do not provide LC-HUFA. We have previously observed the high levels of 18:3n-3 associated with these oils to complicate interpretation of fillet n-3 content with respect to the n-3 LC-HUFA of greatest functional nutritional value to human consumers (15). In this sense, corn oil serves as an excellent negative control with respect to n-3 FA content and was selected for its utility from a hypothesis-testing perspective. Proximate analyses of triplicate diet samples were conducted according to standard methods for analysis of animal feeds (30) for moisture (Official Method 930.15), crude protein (Official Method 954.01), and ash (Official Method 942.05). Crude lipid was determined gravimetrically according to the method of Folch *et al.* (31). Crude lipid samples were reserved for subsequent FA analysis (see section on FA analyses). Mean diet composition was confirmed as 9.6% moisture, 45.2% crude protein, 14.5% crude lipid, and 7.4% ash (dry matter, Table 1).

Experimental design and feeding trial. Before the study, feed-trained sunshine bass were obtained as fingerlings (Keo Fish Farms, Inc., Keo, AR, USA) and reared in-house to submarket size (age 1 [~14 months], 347 ± 8.6 g, mean individual weight ± SEM) on standard, commercial sunshine bass grow-out diets. The fish were then stocked into a water recirculation system consisting of 30 270-L tanks and associated mechanical and biological filtration units at six fish per tank.

The feeding trial (28-wk duration) consisted of five feeding regimen treatment groups using the CO diet, the MO diet, or temporal combinations of both diets (Fig. 2). To establish similar nutritional status and initial FA profiles, the fish were fed assigned diets for a 16-wk baseline period. After 16 wks, one fish was removed from each replicate tank, euthanized, and sampled to determine baseline FA profile. After collection of the baseline samples, the remaining 12 wk of the experimental period were divided into three 4-week intervals for finishing diet implementation. The positive control treatment was fed the MO diet and the negative control treatment was fed the CO diet for the duration of the 28-wk trial. Three interval treatment groups were initially fed the production diet, followed by implementation of the finishing diet at 4-, 8-, or 12-wk before harvest. The five treatments established over the 28-wk feeding trial—0 (CO control), 4, 8, 12, and 28 (MO control) wk of MO diet use—were each randomly assigned to six replicate tanks, with tanks serving as experimental units ($N = 6$). Fish were fed the appropriate diets every other day to apparent satiation, and any pellets remaining 1 h after feeding were removed and enumerated.

Temperature, dissolved oxygen (YSI Model 55 Oxygen Meter, Yellow Springs, OH, USA), and pH (WTW Model PH315i Handheld pH Meter, Weilheim, Germany) were mea-

TABLE 1
Formulation and Proximate Composition of Experimental Diets

	Dietary formulation	
	CO (production) diet	MO (finishing) diet
Ingredient[a]		
Menhaden meal[b]	500.0	500.0
Menhaden oil	—	85.0
Corn oil	85.0	—
Wheat flour	336.8	336.8
Corn gluten	64.2	64.2
Mineral premix	1.0	1.0
Vitamin premix	6.0	6.0
Vitamin C (Stay-C 35%)	1.0	1.0
Choline (70%)	6.0	6.0
Proximate composition[c]		
Moisture	9.7	9.5
Protein	44.7	45.6
Lipid	14.4	14.5
Ash	8.5	6.2

[a]Expressed as g kg^{-1} feed, dry-matter basis.
[b]Typically contains 7.7% crude lipid (44); sufficient to meet LC-HUFA requirements of sunshine bass at present inclusion rate.
[c]Expressed as percent dry matter (except moisture).

TABLE 2
Total Lipid FA Composition of Experimental Diets[a]

	Dietary formulation	
	CO (production) diet	MO (finishing) diet
14:0	1.98	9.57
15:0	0.18	0.78
16:0	15.26	23.97
17:0	0.18	0.48
18:0	2.95	4.72
20:0	0.38	0.42
22:0	0.11	0.16
Saturates	21.06	40.09
16:1n-7	1.98	11.18
18:1n-9	23.84	8.50
18:1n-7	1.32	3.28
20:1n-9	0.42	0.67
22:1n-11	0.20	0.13
22:1n-9	0.11	0.09
Monoenes	27.86	23.86
18:2n-6	42.19	5.42
18:3n-6	0.03	0.19
20:2n-6	0.07	0.18
20:3n-6	0.05	0.18
20:4n-6	0.52	1.50
n-6	42.86	7.46
18:3n-3	0.98	1.41
18:4n-3	0.27	1.77
20:3n-3	0.02	0.11
20:4n-3	0.12	1.04
20:5n-3	3.03	11.80
22:5n-3	0.52	2.28
22:6n-3	3.27	10.11
n-3	8.21	28.53
PUFA[b]	51.08	36.00
LC-HUFA[c]	7.53	27.03
n-3:n-6	0.19	3.82

[a]Mean FA values (relative mol% of total FAME) of triplicate samples. Totals and ratios of relevant FA classes are also provided.
[b]Includes FA with double bonds ≥2.
[c]Includes FA with carbon chain length ≥20 and double bonds ≥3.

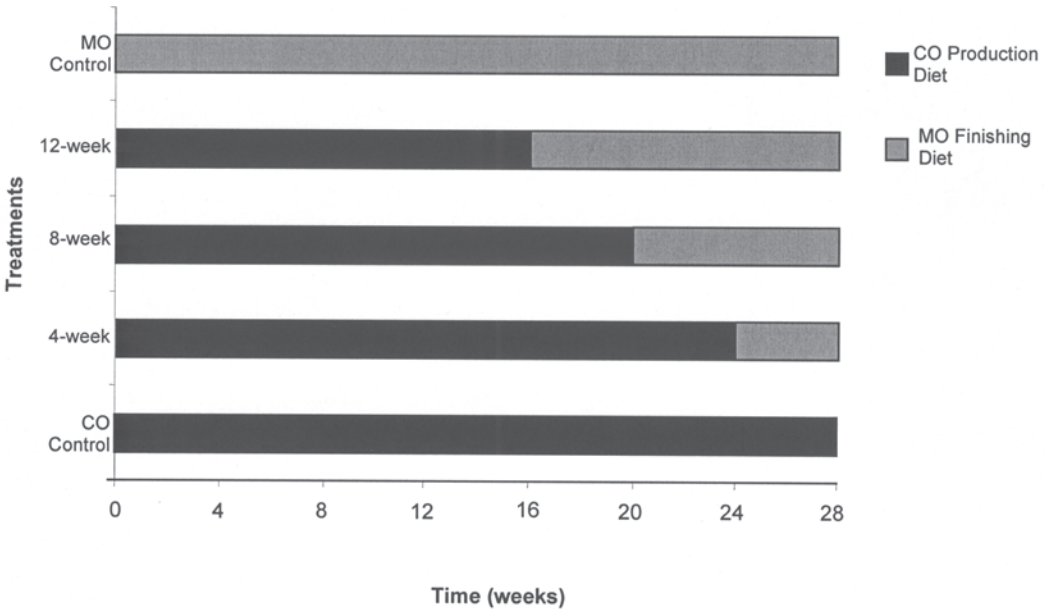

FIG. 2. Schematic representation of feeding regimen treatments with respect to timing of finishing diet implementation.

sured periodically and maintained at 24.0 ± 0.5ºC, 7.7 ± 0.2 mg L^{-1}, and 7.5 ± 0.3, respectively. Alkalinity (mg $CaCO_3$ L^{-1}), ammonia (mg NH_3-N L^{-1}), nitrite (mg NO_2-N L^{-1}), and nitrate (mg NO_3-N L^{-1}) were measured weekly (DR/2010 spectrophotometer and digital titrator, Hach Company, Loveland, CO, USA). All water quality parameters were maintained within ranges suitable for sunshine bass culture (32). All culture and husbandry methods, as well as euthanasia and sample collection procedures described below, were conducted under the direction and approval of the Southern Illinois University Institutional Animal Care and Use Committee, protocol No. 04-016.

Harvest, sample collection, and production performance. Fish were harvested from tanks, weighed, euthanized by single pithing, and immediately packed in ice before processing. Livers and intraperitoneal (IP) fat masses were dissected from the viscera to calculate hepatosomatic (HSI; liver weight/whole body weight*100) and liposomatic (IP fat weight/whole body weight*100) indices. Survival, percent weight gain ([average individual $weight_{final}$/average individual $weight_{initial}$]*100), and food conversion ratio (FCR; weight of food fed /weight gained) were calculated for each tank.

Fillets were harvested by one of four designated filleters, and dressout percentages were calculated as the ratio of the weight of skinless, boneless, J-cut fillets (without belly flap) to total weight of fish. One fillet from each fish was packaged in sterile, polyethylene bags (Whirl-pak®, Nasco, Fort Atkinson, WI, USA) and stored frozen (–80ºC) before proximate and FA analyses.

Proximate composition of fillet meat. Fillet samples were analyzed using standard methods (30) for meat products to determine percent moisture (Official Method 950.46), crude protein (Official Method 981.10), and ash (Official Method 920.153). Crude lipid content was determined using the method of Folch *et al.* (31). Crude lipid samples were reserved for subsequent FA analysis.

FA analyses. Crude lipid samples were subjected to acid-catalyzed transmethylation performed overnight at 50ºC as described previously by Christie (33). The resultant FAME were separated using a Shimadzu GC-17A gas chromatograph (Shimadzu Scientific Instruments**,** Kyoto, Japan) equipped with a flame ionization detector (FID) fitted with a permanently bonded polyethylene glycol, fused silica capillary column (Omegawax 250, 30 m × 0.25 mm i.d., 0.25 μm film). The injection volume was 1.0 μL, helium was the carrier gas (30 cm/s, 205ºC), and the injector temperature was 250°C. A split-less injection technique (100:1) was used, and the temperature program was as follows: 50°C held for 2 min, increased to 220°C at 4°C/min, and held at 220°C for 15 min. Individual FAME were identified by reference to external standards (Supelco 37 Component FAME Mix, PUFA-1, and PUFA-3; Supelco, Bellefonte, PA, USA). All solvents used were of HPLC grade and obtained from Sigma Diagnostics Inc. (St. Louis, MO, USA).

Evaluation of FA turnover and the dilution model. The dilution equation suggested as a generalized model of FA turnover in fishes (14,26–28) may be expressed as

$$P_T = P_R + (P_R - P_I)/(Q_T/Q_I)$$

where P_T is the percentage of a FA in a test fillet at time T following dietary change, P_I and P_R are the percentages of the same FA initially (before dietary change) and in a reference fillet (fed the initial diet throughout), and Q_I and Q_T represent total lipid (total FA) initially and at time T, respectively. To test the suitability of the dilution model for sunshine bass, we compared actual FA composition with the fillet composition predicted by the generalized dilution model using baseline and

TABLE 3
Total Lipid FA Composition of Baseline Fillet Samples (Wk 16)[a]

	Feeding regimen				
FA	CO control	4-wk	8-wk	12-wk	MO control
14:0	2.7 ± 0.2[b]	2.3 ± 0.2[b]	2.3 ± 0.2[b]	2.6 ± 0.2[b]	3.7 ± 0.2[a]
16:0	19.2 ± 0.4[b]	20.1 ± 0.4[b]	20.4 ± 0.4[b]	19.5 ± 0.4[b]	21.3 ± 0.4[a]
18:0	3.4 ± 0.3[a]	3.8 ± 0.3[a]	3.9 ± 0.3[a]	3.4 ± 0.3[a]	3.5 ± 0.3[a]
Saturates[b]	31.2 ± 0.8[b]	32.5 ± 0.8[b]	32.5 ± 0.8[b]	30.7 ± 0.8[b]	37.2 ± 0.8[a]
16:1n-7	6.0 ± 0.5[b]	5.4 ± 0.5[b]	3.6 ± 0.5[c]	5.5 ± 0.5[b]	8.1 ± 0.5[a]
18:1n-9	26.3 ± 1.3[a]	23.9 ± 1.3[a]	24.5 ± 1.3[a]	27.2 ± 1.3[a]	24.1 ± 1.3[a]
18:1n-7	2.3 ± 0.1[b]	2.2 ± 0.1[b]	2.1 ± 0.1[b]	2.2 ± 0.1[b]	2.9 ± 0.1[a]
20:1n-9	1.4 ± 0.1[a]	1.3 ± 0.1[a]	1.2 ± 0.1[a]	1.4 ± 0.1[a]	1.5 ± 0.1[a]
Monoenes[c]	36.2 ± 1.8[a]	33.1 ± 1.8[a]	31.8 ± 1.8[a]	36.6 ± 1.8[a]	37.0 ± 1.8[a]
18:2n-6	19.5 ± 0.6[a]	19.0 ± 0.6[a]	20.4 ± 0.6[a]	20.9 ± 0.6[a]	10.0 ± 0.6[b]
20:2n-6	0.9 ± 0.0[a]	1.0 ± 0.0[a]	0.9 ± 0.0[a]	0.9 ± 0.0[a]	0.6 ± 0.0[b]
20:4n-6	1.2 ± 0.1	1.6 ± 0.1[a]	1.4 ± 0.1[a]	1.1 ± 0.1[a]	1.5 ± 0.1[a]
n-6[d]	24.3 ± 0.6[a]	23.8 ± 0.6[a]	24.9 ± 0.6[a]	25.7 ± 0.6[a]	15.3 ± 0.6[b]
18:3n-3	1.1 ± 0.1[a]	1.0 ± 0.1[a]	0.9 ± 0.1[a]	1.0 ± 0.1[a]	1.1 ± 0.1[a]
20:5n-3	5.0 ± 0.4[b]	5.4 ± 0.4[b]	5.1 ± 0.4[b]	4.3 ± 0.4[b]	7.4 ± 0.4[a]
22:5n-3	1.2 ± 0.1[b,c]	1.3 ± 0.1[b]	1.2 ± 0.1[b,c]	1.0 ± 0.1[c]	1.6 ± 0.1[a]
22:6n-3	7.7 ± 1.1[a]	9.5 ± 1.1[a]	9.9 ± 1.1[a]	6.7 ± 1.1[a]	9.7 ± 1.1[a]
n-3[e]	4.2 ± 0.1[c]	4.6 ± 0.1[b]	4.2 ± 0.1[b,c]	3.8 ± 0.1[c]	5.0 ± 0.1[a]
PUFA[f]	28.5 ± 0.5[a]	28.3 ± 0.5[a]	29.1 ± 0.5[a]	29.6 ± 0.5[a]	20.4 ± 0.5[b]
LC-HUFA[g]	3.9 ± 0.2[b]	4.5 ± 0.2[a]	4.2 ± 0.2[a,b]	3.6 ± 0.2[b]	4.6 ± 0.2[a]
n-3:n-6	0.2 ± 0.0[b,c]	0.2 ± 0.0[b]	0.2 ± 0.0[b,c]	0.2 ± 0.0[c]	0.3 ± 0.0[a]
Bioactive LC-HUFA[h]	1.7 ± 0.1[b,c]	1.9 ± 0.1[b]	1.7 ± 0.1[b,c]	1.5 ± 0.1[c]	2.4 ± 0.1[a]

[a]Mean values (relative mol% of total FAME) ± SEM of predominant muscle FA (≥1%) from replicate groups (1 fish subsampled from each of 6 replicate tanks per regimen treatment, $N = 6$) of sunshine bass according to feeding regimen. Totals and ratios of relevant FA classes and crude lipid composition are also provided. Note all treatment groups were fed the CO diet during the baseline period, except the MO control group, which was fed the MO diet throughout the feeding trial.
[b]Also includes 15:0, 17:0, 20:0, and 22:0.
[c]Also includes 22:1n-11 and 22:1n-9.
[d]Also includes 18:3n-6 and 20:3n-6
[e]Also includes 18:4n-3, 20:3n-3, and 20:4n-3.
[f]FA with double bonds ≥2.
[g]FA with carbon chain length ≥20 and double bonds ≥3.
[h]Defined by authors as 20:4n-6, 20:5n-3, and 22:6n-3.

harvest data from the MO control and 12-wk MO treatments. The model was previously shown to be relatively robust to using whole body masses as surrogate measures of Q_T and Q_I when total adiposity changed little over time (14). As the proximate composition of fillets did not differ among treatment groups or change considerably over the 12-wk finishing period, we substituted average baseline body mass (Q_B) and average mass at harvest (Q_H) for Q_I and Q_T, respectively.

Statistical analyses. Production performance, fillet proximate composition, and FA profile data were subjected to ANOVA within the GLM framework of the Statistical Analysis System, version 9.1 (SAS Institute, Cary, NC, USA) to determine if differences existed among feeding regimen treatment groups. Actual and predicted FA profiles of the 12-wk MO group were similarly analyzed using ANOVA to determine if observed FA abundance differed significantly from predicted levels. For all statistical analyses, individual tanks were used as experimental units, and variation among tanks within treatments was used as the experimental error to test for significance. Differences were considered significant at $P \leq 0.05$.

RESULTS

Feeding the CO diet during the baseline period significantly altered the FA profile of sunshine bass fillets, specifically reducing the level of n-3 and LC-HUFA in favor of n-6 and PUFA (double bonds ≥2), particularly 18:2n-6 (Table 3). After completion of the 16-wk baseline period, fillets of fish fed the CO diet (including CO control, 4-, 8-, and 12-week MO treatment groups) were significantly lower in 14:0, 16:0, 18:1n-7, 20:5n-3, and total saturates, and higher in 18:2n-6, total n-6, and total PUFA compared to fillets from fish that had been solely fed the MO diet (MO control group). Although some significant differences were observed among the CO fed treatment groups, that is, 16:1n-7, 22:5n-3, total n-3, total LC-HUFA, total bioactive LC-HUFA (20:4n-6, 20:5n-3, and 22:6n-3), and n-3:n-6 ratio; in general, these differences were numerically small and remained statistically distinct from the MO control group.

Implementation of the MO finishing diet during the final 12 wk of the experimental period significantly altered fillet FA composition, partially mitigating the effects of the CO diet on

TABLE 4
Total Lipid FA Composition of Harvest Fillet Samples (Wk 28)[a]

	Feeding regimen				
FA	CO control	4-wk	8-wk	12-wk	MO control
14:0	2.3 ± 0.1[c]	2.6 ± 0.1[b,c]	2.7 ± 0.1[b]	2.8 ± 0.1[b]	3.4 ± 0.1[a]
16:0	19.0 ± 0.3[b]	19.4 ± 0.3[b]	19.6 ± 0.3[b]	19.6 ± 0.3[b]	21.2 ± 0.3[a]
18:0	3.4 ± 0.2[a]	3.5 ± 0.2[a]	3.6 ± 0.1[a]	3.5 ± 0.2[a]	4.0 ± 0.1[a]
Saturates[b]	25.2 ± 0.4[c]	26.1 ± 0.4[b,c]	26.5 ± 0.4[b]	26.8 ± 0.4[b]	29.3 ± 0.4[a]
16:1n-7	5.5 ± 0.2[c]	5.8 ± 0.2[b,c]	6.0 ± 0.2[b,c]	6.3 ± 0.2[b]	7.4 ± 0.2[a]
18:1n-9	24.9 ± 0.6[a]	23.9 ± 0.6[a]	23.2 ± 0.6[a]	24.1 ± 0.6[a]	19.3 ± 0.6[b]
18:1n-7	2.2 ± 0.1[c]	2.4 ± 0.1[b,c]	2.4 ± 0.1[b]	2.5 ± 0.1[b]	2.9 ± 0.1[a]
20:1n-9	1.3 ± 0.0[a]	1.4 ± 0.0[a]	1.3 ± 0.0[a]	1.4 ± 0.0[a]	1.2 ± 0.0[a]
Monoenes[c]	34.2 ± 0.8[a]	33.8 ± 0.8[a]	33.2 ± 0.8[a,b]	34.5 ± 0.8[a]	31.1 ± 0.8[b]
18:2n-6	21.9 ± 0.8[a]	18.8 ± 0.8[b]	17.4 ± 0.7[b]	17.3 ± 0.8[b]	9.4 ± 0.7[c]
20:2n-6	1.0 ± 0.0[a]	0.8 ± 0.0[b]	0.8 ± 0.0[b]	0.8 ± 0.0[b]	0.6 ± 0.0[c]
20:4n-6	1.3 ± 0.1[c]	1.4 ± 0.1[b,c]	1.6 ± 0.1[b]	1.4 ± 0.1[b,c]	2.1 ± 0.1[a]
n-6[d]	24.6 ± 0.7[a]	21.5 ± 0.8[b]	20.2 ± 0.7[b]	20.0 ± 0.7[b]	12.4 ± 0.7[c]
18:3n-3	1.0 ± 0.0[a]	1.0 ± 0.0[a]	1.0 ± 0.0[a]	1.0 ± 0.0[a]	1.1 ± 0.0[a]
20:5n-3	4.8 ± 0.3[c]	5.7 ± 0.3[b,c]	6.3 ± 0.3[b]	6.2 ± 0.3[b]	8.9 ± 0.3[a]
22:5n-3	1.2 ± 0.0[c]	1.4 ± 0.0[b]	1.5 ± 0.0[b]	1.4 ± 0.0[b]	1.9 ± 0.0[a]
22:6n-3	8.3 ± 0.6[c]	9.7 ± 0.6[b,c]	10.2 ± 0.6[b]	9.6 ± 0.6[b,c]	13.9 ± 0.5[a]
n-3[e]	16.0 ± 0.9[c]	18.7 ± 0.9[b]	20.1 ± 0.8[b]	19.0 ± 0.9[b]	27.2 ± 0.8[a]
PUFA[f]	40.7 ± 0.6[a]	40.1 ± 0.6[a]	40.3 ± 0.6[a]	39.0 ± 0.6[a]	39.5 ± 0.6[a]
LC-HUFA[g]	16.2 ± 1.0[c]	18.9 ± 1.0[b,c]	20.3 ± 1.0[b]	19.1 ± 1.0[b]	27.6 ± 0.9[a]
n-3:n-6	0.7 ± 0.1[c]	0.9 ± 0.1[b,c]	1.0 ± 0.1[b]	1.0 ± 0.1[b]	2.3 ± 0.1[a]
Bioactive LC-HUFA[h]	14.4 ± 0.9[c]	16.8 ± 1.0[b,c]	18.1 ± 0.9[b]	17.1 ± 0.9[b,c]	24.9 ± 0.9[a]

[a]Mean values (relative mol% of total FAME) ± SEM of predominant muscle FA (≥1%) from replicate groups (5 fish sampled from each of 6 replicate tanks per regimen treatment, $N = 6$) of sunshine bass according to feeding regimen. Totals and ratios of relevant FA classes are also provided.
[b]Also includes 15:0, 17:0, 20:0, and 22:0.
[c]Also includes 22:1n-11 and 22:1n-9.
[d]Also includes 18:3n-6 and 20:3n-6
[e]Also includes 18:4n-3, 20:3n-3, and 20:4n-3.
[f]FA with double bonds ≥2.
[g]FA with carbon chain length ≥20 and double bonds ≥3.
[h]Defined by authors as 20:4n-6, 20:5n-3, and 22:6n-3.

fillet FA profile (Table 4). Although complete restoration (statistically equivalent to MO control group) was not observed, fillet 18:2n-6, 20:2n-6, 22:5n-3, total n-6, and total n-3 FA were significantly altered to reflect a MO-associated FA profile after a finishing period of 4 wk. After an 8-wk finishing period, significant restorative effects were observed for 14:0, 18:1n-7, 20:4n-6, 20:5n-3, 22:6n-3, total saturates, LC-HUFA, bioactive LC-HUFA, and n-3:n-6 FA ratio (Table 4). Fillet 16:1n-7 content also was observed to change following finishing diet implementation; however, significance was only noted after a finishing period of 12 wk.

Comparison of actual FA profile of fillets from the 12-wk MO group with the profile predicted by the model indicated simple dilution adequately described turnover for a majority of the dominant fillet FA; however, significant deviation from predicted values were noted (Fig. 3). Actual abundance greater than predicted values was observed for 20:2n-6, n-3, PUFA, LC-HUFA, and bioactive LC-HUFA, indicating selective retention of these FA classes. Conversely, saturates were less abundant than predicted by the model, indicating selective catabolism of these FA.

Equivalent production performance was observed among all treatment groups (Table 5). Survival (100 ± 0%, mean ± SEM), weight gain (206 ± 19%), food conversion ratio (1.7 ± 0.1), dressout (28 ± 0.4%), hepatosomatic (2.8 ± 0.1) and liposomatic (3.7 ± 0.5) indices were statistically equivalent among dietary groups and within acceptable ranges for sunshine bass production performance. Fillet moisture (75.3 ± 1.8%), crude protein (21.3 ± 1.2%), crude lipid (2.5 ± 0.5%), and ash (1.2 ± 0.1%) content were not affected by dietary treatment.

DISCUSSION

Replacing MO with CO as the primary lipid source in sunshine bass diet yielded fillets with distinctly different FA profiles (Table 3). However, finishing with a MO-based diet offered partial compensation for CO-associated reductions in beneficial fillet FA (Table 4). Use of the CO diet increased fillet n-6 and PUFA content at the expense of n-3 and LC-HUFA. These fillets were significantly lower in saturates and higher in PUFA, but this is primarily due to an approximately twofold increase in 18:2n-6 content. High levels of 18:2n-6 and reduced n-3 FA incorporation skewed the n-3:n-6 FA ratio heavily in favor of n-6 FA. This suboptimal ratio, coupled with markedly lower

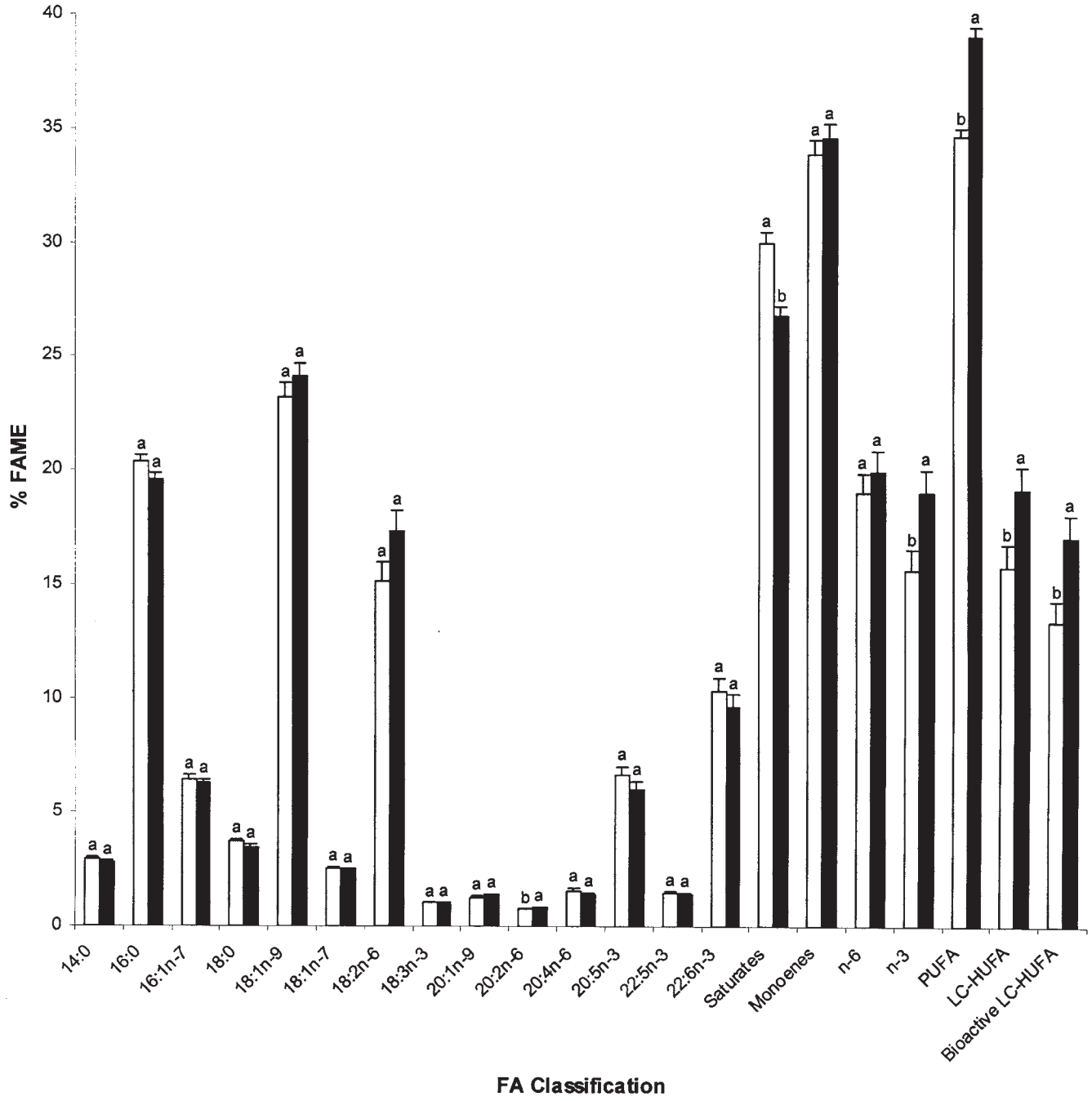

FIG. 3. Comparison of actual FA composition (relative mol%) of sunshine bass fillets (black bars) and predicted composition (white bars) based on the dilution model $P_{12} = P_R + (P_R - P_0)/(Q_H/Q_B)$ where P_0 and P_{12} are the percentages of a FA in the fillet before and after 12 wk of finishing diet implementation, P_R is the percentage of the FA in reference fillets from the MO control group, and Q_B and Q_H are the average body mass after completion of the baseline period and at harvest, respectively. Error bars represent ±SEM, and columns within FA classifications with common superscripts are not significantly different ($P \geq 0.05$).

levels of bioactive LC-HUFA, indicates these fillets would be of reduced functional nutritional value to human consumers. Implementation of the MO diet at the end of the production cycle had a restorative effect on fillet FA composition (Table 4), partially reversing the effects of using the CO diet. Significant restoration of endogenous n-3 FA was achieved within 4 wk of feeding the MO diet, and a significant increase in LC-HUFA and a beneficial shift in n-3:n-6 FA ratio was observed after 8 wk.

Finishing diets have been successfully used to augment MO-associated FA content of turbot (22) and Atlantic salmon (24,25) fed diets containing plant-derived lipids. Although the

TABLE 5
Production Performance and Fillet Proximate Composition[a,b]

Parameter	Feeding regimen				
	CO control	4-wk	8-wk	12-wk	MO control
Production performance					
Survival	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Weight gain (%)	197 ± 19	199 ± 19	233 ± 19	212 ± 19	187 ± 19
Initial weight (g)	362 ± 19	343 ± 19	329 ± 19	333 ± 19	368 ± 19
Baseline weight (g)	490 ± 40	469 ± 40	482 ± 40	575 ± 40	608 ± 4
Harvest weight (g)	710 ± 23	660 ± 23	705 ± 23	707 ± 23	687 ± 23
FCR	1.7 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.8 ± 0.1
Dressout (%)	28 ± 0.4	27 ± 0.4	28 ± 0.4	27 ± 0.4	29 ± 0.4
HSI	3.0 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.6 ± 0.1
LSI	4.8 ± 0.5	3.8 ± 0.5	3.3 ± 0.5	3.1 ± 0.5	3.2 ± 0.5
Fillet proximate composition[c]					
Moisture (%)	75.9 ± 1.8	76.0 ± 1.8	73.4 ± 1.8	73.1 ± 1.8	78.3 ± 1.8
Protein (%)	21.7 ± 1.2	22.2 ± 1.2	21.8 ± 1.2	20.2 ± 1.2	20.6 ± 1.2
Lipid (%)	2.7 ± 0.5	2.2 ± 0.5	2.5 ± 0.5	3.2 ± 0.5	2.0 ± 0.5
Ash (%)	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1

[a]No significant differences were noted among feeding regimens for any of the parameters ($P > 0.05$).
[b]Means ± SEM, 5 fish sampled from each of 6 replicate tanks per regimen treatment ($N = 6$).
[c]Percent composition may not sum to 100 due to analytical margins of error and rounding.

negative effects of plant-derived lipid on fillet FA profile were partially reversed in these studies, the finishing period necessary to elicit significant compositional changes was longer (8–20 wk vs. 4–8 wk) for these species relative to our results. Moreover, many of these studies used diets containing in excess of 30% crude lipid, roughly twice the lipid content of the feeds we used to elicit similar composition changes.

Asymptotic dilution models suitably described FA turnover in Atlantic salmon (14,26), red seabream (27), and brown trout *Salmo trutta* (28), with rapid changes in fillet composition observed shortly after dietary modification giving way to diminishing effects over the growth period (Fig. 1). However, Jobling (14,26) cautioned the model, though apparently well-suited to medium-fat species, may not be appropriate for lean-fleshed fish. Structural phospholipids are dominant components of the lipid fraction in lean tissues, and it was suggested the relative unresponsiveness of the polar lipid fraction may cause FA turnover to deviate from the dilution model in these species (14,26). We found simple dilution accurately predicts tissue composition for most predominant FA, which are apparently deposited within muscle tissue with little-to-no specificity with respect to final tissue composition. However, deviations from the model were also noted, suggesting selective metabolism of certain FA classes (27). Specifically, we found total n-3 FA, PUFA, LC-HUFA, and especially bioactive LC-HUFA in greater than expected abundance, suggesting preferential retention of these FA; whereas actual levels of saturates were below predicted levels, suggesting preferential catabolism or deposition in other body compartments. Apparent processes of selective retention are further supported by differences in turnover rates observed for n-3, LC-HUFA, and bioactive LC-HUFA. Selective retention would imply faster turnover rates for FA selected for deposition compared to rates for other FA. Comparing the FA profiles of fillets from the CO control and the 4-wk MO treatments, the absolute percent change achieved after 4 wk of finishing diet use is consistently higher (mean 17%) for n-3, LC-HUFA, and bioactive LC-HUFA than other FA (mean 8%). Finally, it is important to note the preferentially retained FA classes were not those found in the greatest abundance in sunshine bass fillet polar fractions (namely 16:0, 18:1n-9, and 18:2n-6 [34]), and thus the selective processes we observed do not appear to be artifacts of greater total polar lipid. Accordingly, our results strongly suggest (1) n-3, PUFA, LC-HUFA, and bioactive LC-HUFA are selectively retained within sunshine bass muscle tissue; and (2) this process is functionally independent of changes in structural phospholipid elements during growth of muscle tissue.

Many fish species, including white bass *M. chrysops* (35), striped bass *M. saxatilis* (36), and their hybrids (37), have dietary requirements for 20:4n-6, 20:5n-3, and 22:6n-3 due to limited activity of appropriate enzymes to produce sufficient amounts of these bioactive LC-HUFA from 18-carbon precursors (38). Several groups have suggested limited endogenous and exogenous availability obliges moronids to conserve these FA (35,39–41). Our observation of bioactive LC-HUFA levels in excess of predicted values supports this hypothesis. The FA dilution model is adequate for estimating gross changes in fillet composition of fish species following dietary change; however, it does not appear to fully describe the pattern of FA turnover in lean-fleshed fish such as sunshine bass. Further experimentation carefully designed to address interspecific differences in biology, differences in gross dietary lipid content, and culture conditions is necessary to unequivocally define temporal patterns of FA change in fishes.

Nutritional and medical communities continue to recommend increasing human consumption of high-quality seafood to improve and maintain public health (42). With global seafood consumption at record highs and many food-grade

fisheries apparently static or in decline (1), aquaculture must assume a greater role in meeting seafood demand (43). Unfortunately, limited availability of high LC-HUFA-content feedstuffs places demands for increased quantity and quality of aquaculture products in juxtaposition. Partial or complete replacement of marine-derived lipid in aquafeeds reduces production costs, relieves harvest pressure on feed-grade fisheries, limits transfer and accumulation of contaminants, and reduces the environmental "footprint" of aquaculture. However, utilization of alternative lipids throughout the production cycle significantly alters fillet FA profile and reduces the functional nutritional value of cultured seafood products. By implementing finishing diets, aquaculturists may preserve or enhance the marketability of their products while decreasing production cost and dependence on finite resources. Although our results are promising, full realization of these goals will require the development of nutritional strategies to optimize fillet FA profile. Refining our understanding of FA turnover in fishes will provide the scientific foundation for successful application of these principles in aquaculture, and ultimately provide for optimal nutrition and management of aquatic livestock.

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^{1}H and ^{13}C NMR Studies of Melon and Head Blubber of the Striped Dolphin (*Stenella coeruleoalba*)

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ABSTRACT: Intact portions of melon, the echolocation organ of the striped dolphin (*Stenella coeruleoalba*), and the corresponding raw oils were analyzed by means of one- and two dimensional ^{1}H and ^{13}C NMR techniques. For comparative purposes the tissue and the raw oil of head blubber were also examined. Complete assignments of the spectra were obtained. Furthermore, dynamics of the lipid components was investigated by means of ^{13}C NMR spin lattice relaxation time (T_1). Analysis of the data revealed that lipid molecules in the tissue compartments experience a liquid-like microenvironment and that T_1 values depend on the lipid composition and/or organization in the intact tissue framework. In particular, a dependence of the T_1 values on the wax esters content in melon intact tissues was found. A possible correlation between dynamic parameters and sound propagation properties has been hypothesized.

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It is well known that odontocetes echolocate by producing clicking sounds and then receiving and interpreting the resulting echo. The melon, the rounded region of the dolphin's forehead, is instrumental to echolocation; it has been hypothesized to act as an acoustic lens that focuses the sound waves, produced in the monkey lips/dorsal bursa (MLDB) complex, into a beam, which is projected forward, into the aqueous medium in front of the animal (1).

The melon of odontocetes contains very unusual lipid components, referred to as "acoustic fats," that differ from those of the blubber and other body fats (2,3). In the case of adult individuals of *Stenella coeruleoalba* (order Cetacea, suborder Odontoceti, family Delphinidae), morphological analysis has shown that the melon is mainly composed by an adipose tissue, with closely related voluminous cells, and a muscle component that progressively fades from the basal toward the upper sections (4). The lipid components belong to two main classes: triacylglycerols (TAG) and wax esters (WE), composed by high concentrations of iso-branched fatty acids and fatty alcohols, among which isovalerate (iso5:0) is the most abundant. The remaining fatty components are linear with chain of C10–C18 average length, and have a low degree of unsaturation (2–4). It has been demonstrated that the relative amounts of fatty molecules change greatly within the organ (5). Comparative studies on blubber fats have shown that they lack WE, exhibit a lower concentration of iso5:0, a higher unsaturation degree, and are composed of longer chain acids (C14–C22) (3,6).

Several investigations have suggested that the acoustic properties of the melon of odontocetes are due to its peculiar and heterogeneous lipid molecular composition and organization in the tissue framework (5,7–9). In this connection, areas with different lipid composition have been shown to exhibit different sound properties (5,7). Furthermore, several studies demonstrated that sound velocity in lipid mixtures from acoustic tissues of porpoises is influenced by changes in the ratio of WE to TAG (5,8), and that short and branched fatty acids have lower sound velocities compared to long- and straight-chain fatty acids (8,9). In particular, the *sn*-1,3 position of iso5:0 in TAG was suggested to be critical for modulating density and compressibility, which are properties correlated with sound transmission (6,7,10).

The above statements drive to the consequence that the molecular composition and organization *in situ* may hold clues to the acoustic properties of melon; therefore, studies on these acoustic fats should be performed on intact tissues. Nevertheless, most of the investigations of the acoustic tissues of odontocetes have been so far performed by using invasive techniques involving extraction and fractionation of the molecular components, thus destroying the structural organization of the tissues. NMR spectroscopy offers instead the opportunity of studying heterogeneous lipid mixtures, oils, and depot fats noninvasively and nondestructively (11–17), thus allowing investigations of composition and dynamics of the three-dimensional "packing" of lipids in intact tissues.

In a previous study we used the ^{13}C NMR technique to investigate intact tissue samples of melon of *Stenella coeruleoalba*, providing information on their lipid components at a qualitative and quantitative level (4). In the present work, we extend the use of the NMR spectroscopy to obtain a deeper insight into the lipid components both in intact tissues and in raw oil samples of melon of *Stenella coeruleoalba*. For comparative purposes also samples of tissue and raw oil of head blubber are examined. Compositional and structural details of the lipid matrix are obtained by performing ^{1}H and ^{13}C one-dimensional and two-dimensional heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond cor-

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Abbreviations: HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; TAG, triacylglycerols; WE, wax esters.

relation (HMBC) NMR experiments on the tissues and the corresponding raw oils. Furthermore, to gain an understanding of the dynamic properties of the lipid components in the intact tissue, where the three-dimensional arrangement is preserved, ^{13}C spin-lattice relaxation times (T_1) are measured in samples of melon and blubber tissues.

EXPERIMENTAL PROCEDURES

Samples preparation. Melon and head blubber tissue samples were kindly donated by the Department of Cytomorphology and the Department of Experimental Biology, University of Cagliari, Italy. They were dissected from three adult individuals of striped dolphins, *Stenella coeruleoalba*, found stranded and recently dead along the Mediterranean coasts. The melon and blubber were excised within 24 h from the finding of the stranded dolphins and divided in tissue specimens. For each individual, two samples of melon, obtained at different depths of the organ, were collected. Only one sample of head blubber was available. Samples were placed in 10-mm NMR tubes, rapidly frozen at –80°C, and stored before the NMR experiments. Since tissue samples are easily perishable they were examined only once.

The raw oils from samples of melon and of head blubber were obtained by mild pressing of the tissues. Compared to the melon, a larger amount of blubber tissue was needed to obtain an appreciable quantity of oil for NMR experiments.

NMR spectroscopy. ^1H and ^{13}C NMR spectra of intact portions of tissue were recorded on a Varian VXR 300 spectrometer (Palo Alto, CA) equipped with a 10-mm broadband probe at the resonance frequency of 299.93 and 75.42 MHz, respectively. The experiments were run at 25°C, unlocked and without rotation. ^1H spectra were acquired with a sweep width of 4 KHz, and 16 scans, 1-μs pulse width and 2-s delay. Quantitative ^{13}C spectra were acquired using the NOE suppressed, inverse gated proton decoupled technique (Waltz-16), with a 1-s acquisition time, a sweep width of 19 KHz; 500 scans were collected using a 90° pulse angle and a 35-s relaxation delay to allow all the carbon atoms to completely relax. Chemical shifts were externally referenced to tetramethylsilane (TMS; $\delta = 0.00$ ppm).

The raw oils dissolved in $CDCl_3$ (~50:50 vol/vol) were examined in 5-mm NMR tubes on a Varian UNITY INOVA 400 spectrometer at 25°C. The ^1H spectra were obtained at the frequency of 399.94 MHz, with a sweep width of 6 KHz and 16 scans. The ^{13}C spectra were acquired at the frequency of 100.56 MHz with a 2-s acquisition time, using a sweep width of 26 KHz; 500 scans were collected using a 90° pulse angle and a 30-s relaxation delay. Two dimensional ^1H-^{13}C HSQC and HMBC NMR experiments (18,19) were performed at 25°C with sweep widths of 3 KHz and 21.1 KHz in the proton and the carbon dimensions, respectively, 256 increments, 32 scans, and 2 K data points.

^{13}C spin-lattice relaxation times (T_1) were measured using the inversion-recovery (180-τ-90) pulse sequence (20), with τ values ranging from 0.01 to 30 s in 12 steps.

RESULTS AND DISCUSSION

^1H NMR spectra. Figure 1 shows the ^1H NMR spectra of one sample of intact tissue of melon and head blubber of *Stenella* (a and b, respectively) and those of the corresponding raw oils dissolved in $CDCl_3$ (a^1 and b^1). Despite the broadening of the tissue resonances, due to the semisolid nature of samples, the pattern of signals of each tissue is similar to that of the corresponding raw oil, except for the presence of the resonance at approximately 4.7 ppm attributed to water present in the tissue. Since the signal broadening of the tissue spectra makes the resonance assignments difficult, the identification of lipid components was made on the oil spectra according to the literature (11–13). Chemical shift assignments are reported in Table 1. The analysis of the ^1H NMR spectra evidences that all samples exhibit signals mainly from TAG, and, in the case of melon, also from WE. No molecular components such as those of connective and muscle tissues were detected, since their reduced molecular mobility broadens the signals beyond the detection threshold of the NMR spectrometer.

Comparison between the spectra in Figure $1a^1$ and b^1 shows that the oil of melon has a lower unsaturation degree (signals H-1, H-6, and H-12) than that of blubber, the latter being rich in HUFA (highly unsaturated fatty acids) (signals H-7 and H-13). Furthermore, NMR resonances assigned to iso-branched acyl groups in TAG or WE (signals H-9, H-11, H-15, H-17, H-19, and H-21) have lower intensities in blubber oil spectrum than in that of melon. Finally, signal H-5, assigned to the methylene alcoholic group in WE, and signal H-10, ascribed to isovalerate esterified in WE, are absent in the blubber oil spectrum, thus evidencing the lack of wax components.

^{13}C NMR spectra. The ^{13}C NMR spectra of melon and blubber intact tissues together with the corresponding diluted raw oils are shown in Figures 2 and 3, respectively. It is worth noting that the ^{13}C NMR spectra of both tissues exhibit a good resolution, showing general features similar to those of the corresponding oils.

In our previous study (4), chemical shift assignments of the ^{13}C NMR spectrum of the melon tissue were accurately performed only for alkyl carbons, since signals in the carboxyl and olefinic regions were markedly overlapped. In the present work, complete and detailed signal attributions for both melon and blubber spectra were obtained by comparing the tissue and the corresponding oil spectra and with the aid of the literature data (14–17). Furthermore, additional information were obtained by using the HSQC and HMBC NMR techniques. Chemical shift assignments of the ^{13}C spectra melon tissue and oil are reported in Table 2. As to the signals in the carboxyl region, the following detailed attributions were made: signal C-1 originates from waxes esterified with isovaleric acid; signal C-2 arises from acyl groups, other than isovalerate, in TAG *sn*-2 position; signal C-3 from isovalerate in TAG *sn*-1,3 positions. Moreover, in the olefinic region, signals C-5 and C-6, which are well separated in the oil spectrum, were attributed to the olefinic carbons of monounsaturated acyl groups in TAG or WE. The remaining olefinic resonances were assigned to

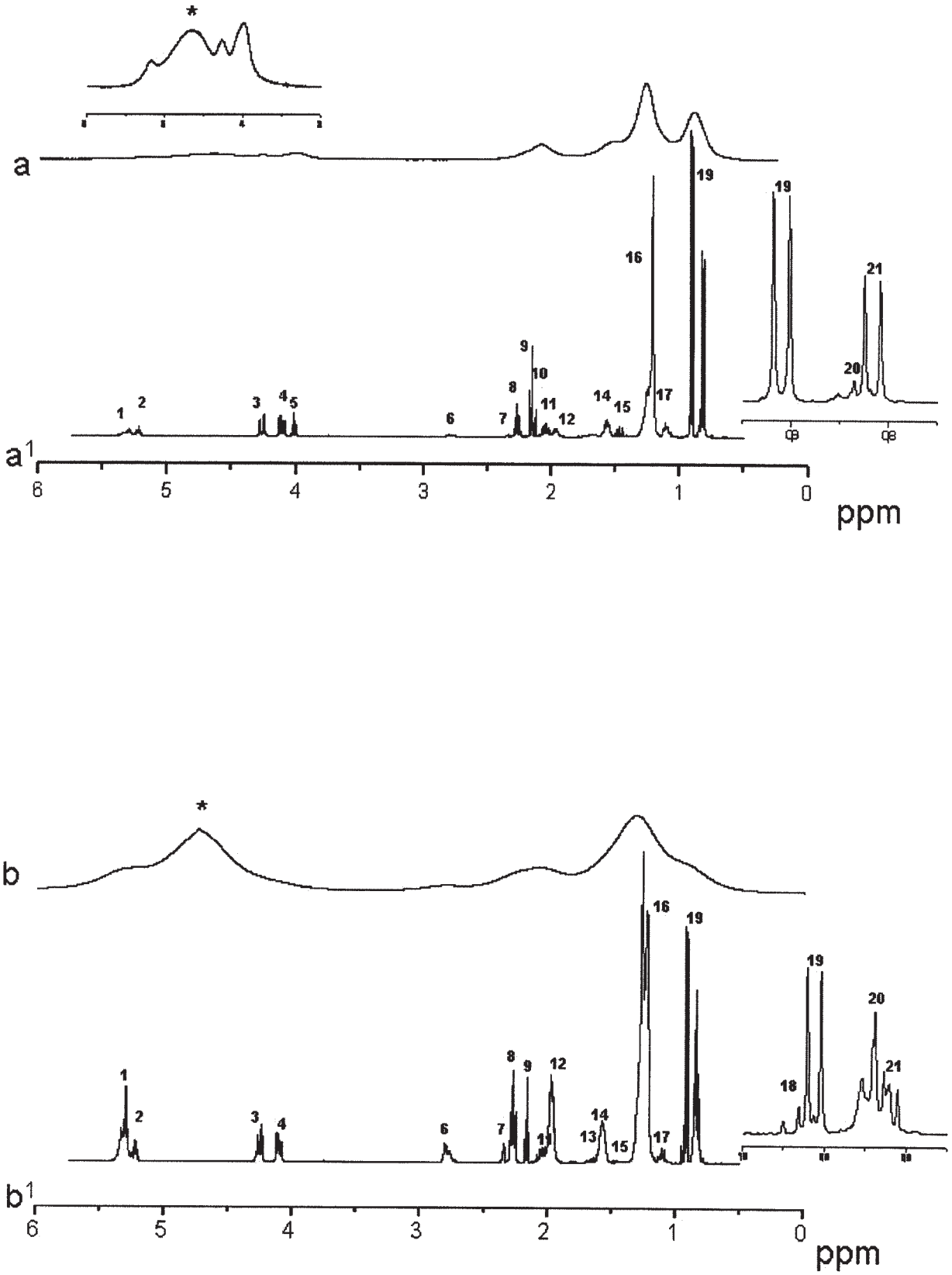

FIG. 1. ^{1}H NMR spectra of samples of intact tissue of melon (a) and head blubber (b) of *Stenella* and the corresponding oils in $CDCl_3$ (a^1 and b^1, respectively). Assignments of the numbered resonances are reported in Table 1. The asterisk indicates the water signal.

TABLE 1
Chemical Shifts Assignments for ^{1}H NMR Spectra of Raw Oils from Melon and Blubber of *Stenella* Diluted in $CDCl_3$

Peak	Compound	Carbon	Chemical shift (ppm)[a]	Multiplicity[b]
1	Unsaturated a.g.[c]	-C<u>H</u>=C<u>H</u>-	5.29	m
2	Glycerol backbone TAG	-C<u>H</u>-COO-	5.22	m
3	Glycerol backbone TAG	-C<u>H</u>$_2$-COO-	4.26	dd
4	Glycerol backbone TAG	-C<u>H</u>$_2$-COO-	4.10	dd
5	Fatty alcohols in WE	-C<u>H</u>$_2$-COO -	4.01	t
6	Polyunsaturated a.g.	-CH=CH-C<u>H</u>$_2$-CH=CH-	2.78	m
7	DHA[d]	-COO-C<u>H</u>$_2$-C<u>H</u>$_2$-CH=	2.34	m
8	All a.g.	-COO-C<u>H</u>$_2$-CH$_2$-	2.26	t
9	Isovaleric a.g. in TAG	-COO -C<u>H</u>$_2$-CH-(CH$_3$)$_2$	2.16	d
10	Isovaleric a.g. in WE	-COO -C<u>H</u>$_2$-CH-(CH$_3$)$_2$	2.13	d
11	Isovaleric a.g.	-COO -CH$_2$-C<u>H</u>-(CH$_3$)$_2$	2.04	m
12	Unsaturated a.g.	-C<u>H</u>$_2$-CH=CH-	1.97	m
13	EPA[e]	-COO-CH$_2$-C<u>H</u>$_2$-	1.65	m
14	All a.g.	-COO-CH$_2$-C<u>H</u>$_2$- CH$_2$-	1.56	m
15	Isobranched a.g.	-CH$_2$-C<u>H</u>-(CH$_3$)$_2$	1.46	m
16	All a.g.	-(CH$_2$)$_n$–	1.21	m
17	Isobranched a.g.	-C<u>H</u>$_2$-CH-(CH$_3$)$_2$	1.10	m
18	All n-3 a.g.	-C<u>H</u>$_3$	0.93	t
19	Isovaleric a.g.	-(C<u>H</u>$_3$)$_2$	0.91	d
20	Linear a.g. except n-3	-C<u>H</u>$_3$	0.84	t
21	Isobranched a.g.	-(C<u>H</u>$_3$)$_2$	0.82	d

[a]Chemical shifts are referenced to TMS.
[b]m = Multiplet, d = doublet, dd = doublet of doublets, t = triplet.
[c]a.g. = Acyl group.
[d]DHA = docosahexaenoic fatty acid.
[e]EPA = eicosapentaenoic fatty acid.

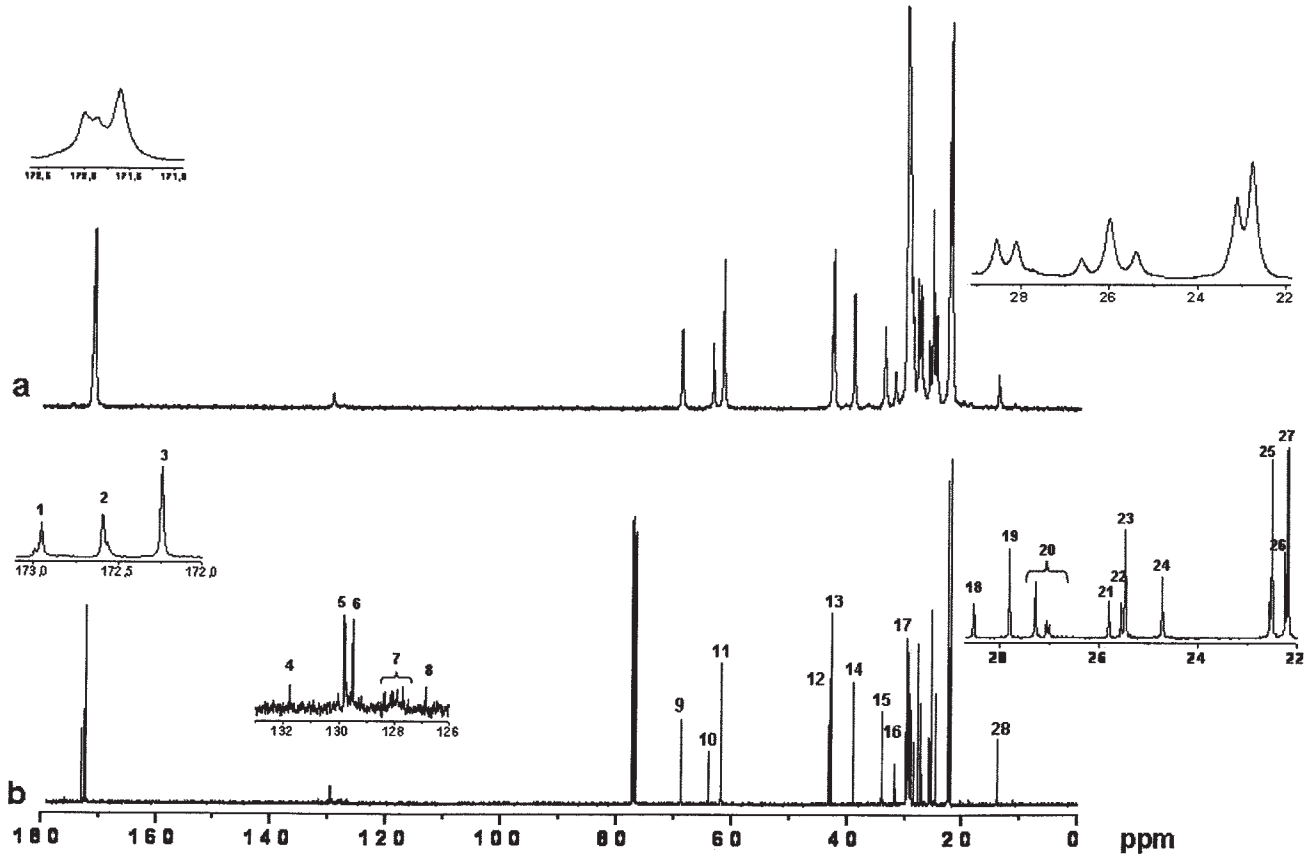

FIG. 2. ^{13}C NMR spectra of intact tissue of melon of *Stenella* (a) and the corresponding oil in $CDCl_3$ (b). Assignments for the numbered resonances are given in Table 2.

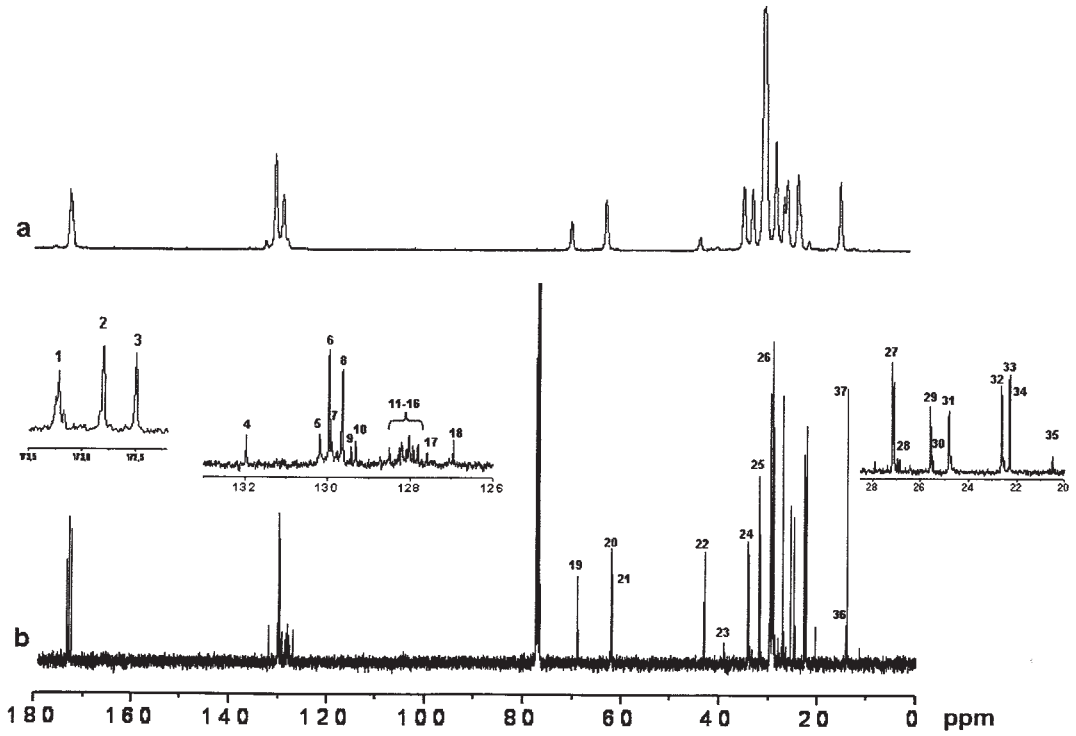

FIG. 3. ^{13}C NMR spectra of intact tissue of head blubber of *Stenella* (a) and the corresponding oil in $CDCl_3$ (b). Assignments for the numbered resonances are given in Table 3.

TABLE 2
Chemical Shifts Assignments for ^{13}C NMR Spectra of the Melon Intact Tissue of *Stenella* and the Corresponding Raw Oil Diluted in $CDCl_3$

Peak	Compound	Carbon	Chemical shift (ppm)[a]	
			Intact tissue	Raw oil
1	Isovaleroyl WE	-CH_2-OO$\underline{C}$-CH_2-	172.00	172.95
2	*sn*-2 TAG	-CH_2-OO$\underline{C}$-CH_2-	171.87	172.60
3	*sn*-1,3 isovaleroyl TAG	-CH_2-OO$\underline{C}$-CH_2-	171.62	172.24
4	All n-3 a.g.[b]	ω3[c]	n.d.	131.78
5	MUFA	-$\underline{C}$H=$\underline{C}$H	130.08	129.81
6	MUFA	-$\underline{C}$H=$\underline{C}$H	130.08	129.50
7	PUFA	-$\underline{C}$H=$\underline{C}$H-	n.d.	127.69
8	All n-3 a.g.	ω4	n.d.	126.86
9	Glycerol backbone TAG	-$\underline{C}$H-OOC-	68.54	68.76
10	Fatty alcohols in WE	-$\underline{C}H_2$-OOC-	64.13	64.08
11	Glycerol backbone TAG	-$\underline{C}$H2-OOC-	62.35	61.84
12	Isovaleric a.g. in WE	-COO-$\underline{C}H_2$-CH-$(CH_3)_2$	43.59	43.33
13	Isovaleric a.g. in TAG	-OOC-$\underline{C}H_2$-CH-$(CH_3)_2$	43.29	42.92
14	Isobranched a.g.	ω3	39.71	38.92
15	All a.g. except isovaleric	-OOC-$\underline{C}H_2$- CH_2-	34.39	34.01, 33.86
16	Linear a.g. except n-3	ω3	32.59	31.79, 31.64
17	All a.g.	-$(CH_2)_n$-	30.18–29.44	29.80–28.84
18	Fatty alcohols in WE	-$\underline{C}H_2$-CH_2-OOC-	28.55	28.53
19	Isobranched a.g.	ω2, ω4	28.11	27.81
20	Unsaturated a.g.	-$\underline{C}H_2$-CH=CH-	n.d.	27.29, 27.06, 27.01
21	Fatty alcohols in WE	-$\underline{C}H_2$-CH_2-CH_2-OOC-	26.62	25.80
22	PUFA	-CH=CH-$\underline{C}H_2$-CH=CH-	25.98	25.55
23	Isovaleric a.g.	-CH_2-$\underline{C}$H-$(CH_3)_2$	25.98	25.47
24	All a.g. except isovaleric	-OO$\underline{C}$-CH_2-$\underline{C}H_2$-	25.40	24.71
25	Linear a.g.	ω2	23.12	22.54, 22.51, 22.49
26	Isobranched a.g.	-$(CH_3)_2$	23.12	22.23
27	Isovaleric a.g.	-$(CH_3)_2$	22.76	22.18
28	Linear a.g.	-CH_3	14.55	13.95

[a]Chemical shifts are referenced to TMS.
[b]a.g. = Acyl groups.
[c]Carbon number three from the methyl end.

TABLE 3
Chemical Shifts Assignments for ^{13}C NMR Spectra of the Blubber Intact Tissue of *Stenella* and the Corresponding Raw Oil Diluted in $CDCl_3$

Peak	Compound	Carbon	Chemical shift (ppm)[a]	
			Intact tissue	Raw oil
1	All a.g.[b] in *sn*-1,3 positions in TAG except isovaleric, EPA, and DHA	-OO<u>C</u>-CH_2-	172.17	173.23, 173.18
2	All a.g. in *sn*-2 position in TAG, except isovaleric and DHA	OO<u>C</u>-CH_2-	172.17	172.80
3	Isovaleric and DHA in *sn*-1,3 positions in TAG	-OO<u>C</u>-CH_2-	172.17	172.50
4	All n-3 a.g.	ω3[c]	131.98	131.97
5	18:2	C13	130.06	130.20
6	Δ9 MUFA	C10	130.06	129.96
7	18:2	C9	130.06	129.91
8	Δ9 MUFA	C9	130.06	129.64
9	DHA	C4	130.06	129.43
10	PUFA	-CH=CH-	130.06	129.32
11	DHA EPA	C17 C15	128.46	128.52
12	DHA	C7	128.46	128.27
13	18:2 DHA	C10 C10, C14	128.46	128.22
14	DHA EPA	C11, C13 C9, C11	128.46	128.04
15	18:2 DHA	C12 C8	128.46	127. 94
16	DHA EPA	C16 C14	128.46	127.82
17	DHA	C5	127.56	127.61
18	All n-3 a.g.	ω4	126.56	126.97
19	Glycerol backbone TAG	-<u>C</u>H-OOC-	69.45	68.84
20	Glycerol backbone TAG	-<u>C</u>H_2-OOC-	62.34	62.05
21	Glycerol backbone *sn*-1,3 isovaleroyl TAG	-<u>C</u>H_2-OOC-	62.34	61.97
22	Isovaleric a.g.	-OOC-<u>C</u>H_2-CH-$(CH_3)_2$	43.17	43.06
23	Isobranched a.g.	ω3	39.61	39.01
24	All a.g. except isovaleric	-OOC-<u>C</u>H_2-	34.25	34.14 33.98
25	Linear a.g. except n-3	ω3	32.46	31.87 31.74
26	All a.g.	-$(CH_2)_n$-	30.17–29.89	29.73–28.95
27	Unsaturated a.g.	-<u>C</u>H_2-CH=CH-	27.67	27.18, 27.12
28	EPA	C4	27.67	26.87
29	PUFA	-CH=CH-<u>C</u>H_2-CH=CH-	26.01	25.59
30	Isovaleric a.g.	-CH_2-<u>C</u>H-$(CH_3)_2$	26.01	25.49
31	All a.g. except DHA	-OOC-CH_2-<u>C</u>H_2-	25.33	24.82
32	Linear a.g. except n-3	ω2	23.17	22.64, 22.61
33	DHA	C3	23.17	22.30
34	Isovaleric a.g.	-$(CH_3)_2$	23.17	22.29
35	All n-3 a.g.	ω2	21.02	20.51
36	All n-3 a.g.	-CH_3	14.48	14.22
37	Linear a.g. except n-3	-CH_3	14.48	14.07

[a]Chemical shifts are referenced to TMS.
[b]a.g. = Acyl groups.
[c]Carbon number three from the methyl end.
[AQ9]

polyunsaturated acyl groups in TAG or WE, among which n-3.

Figures 4 and 5 show the ^1H-^{13}C HSQC and HMBC NMR spectra, respectively, of the melon raw oil. The HSQC experiment correlates protons with their directly attached carbons, whereas the HMBC allows correlations two or three bonds apart.

In Figure 4 cross-peaks between the oxygenated carbons (signals C-9, C-10, and C-11) and the directly attached protons (H-2, H-5, H-3, and H-4) in the respective glyceryl regions can be clearly seen, together with connections of the ^{13}C and ^1H signals of the methylene groups of isovalerate (C-12 and C-13, with H-9 and H-10), and methylene in ω3 position in iso-branched acyl groups (C-14 with H-17). A portion of the

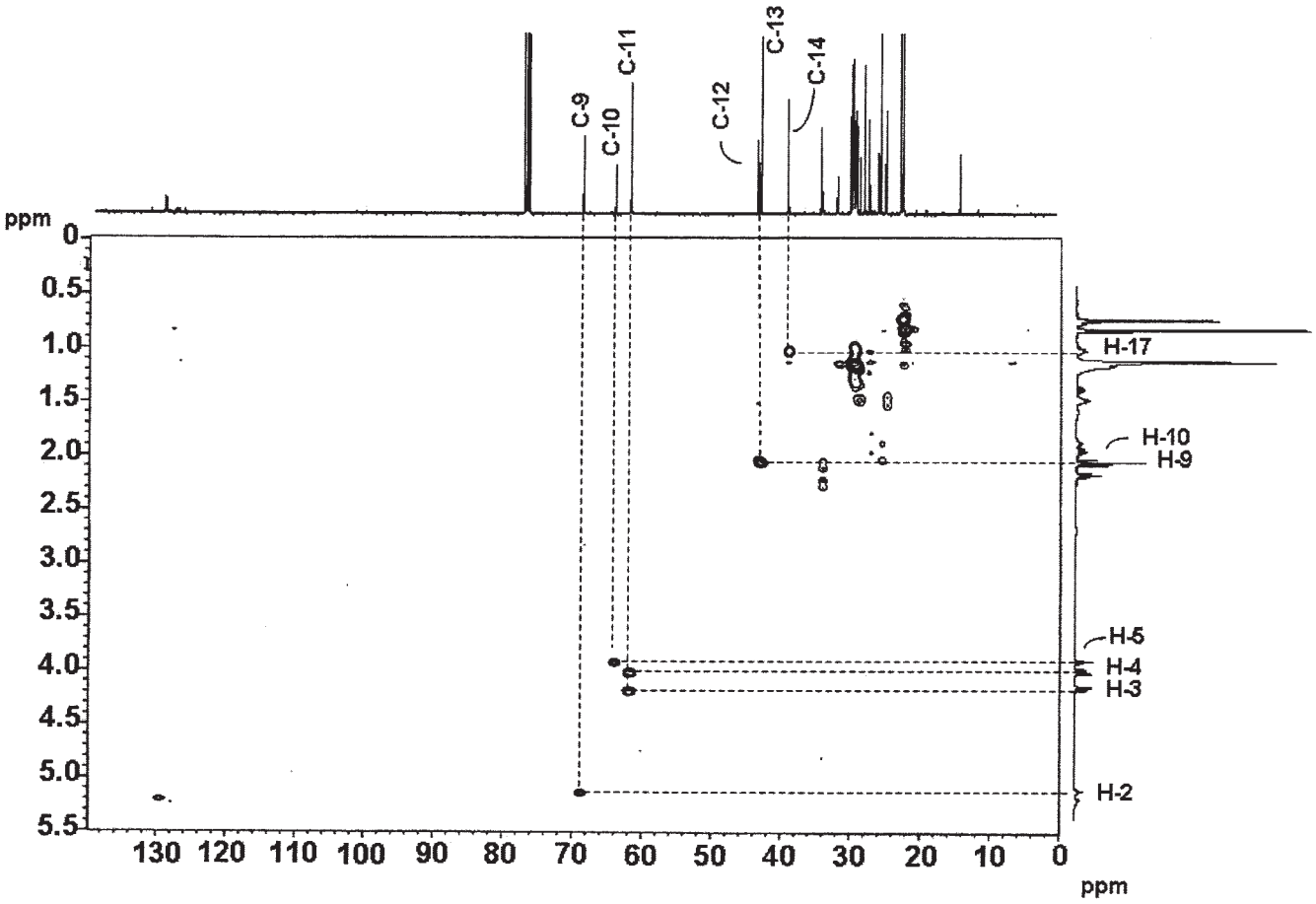

FIG. 4. Heteronuclear single quantum correlation (HSQC) NMR spectrum of raw oil obtained from the melon of *Stenella*.

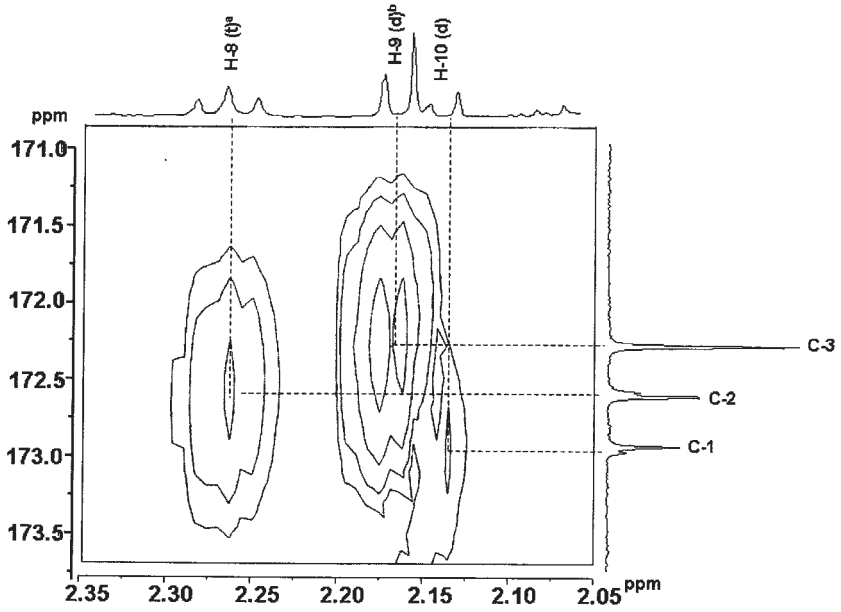

FIG. 5. Portion of the heteronuclear multiple bond correlation (HMBC) NMR spectrum of raw oil obtained from the melon of *Stenella*. (a) t = triplet; (b) d = doublet.

TABLE 4
Relative Percentage Content of Lipids in Intact Portions of Melon (*n* = Sample Size) and Blubber of *Stenella* Obtained from ^{13}C NMR Spectra[a]

	Melon (n = 6)		
	Mean	Range[b]	Blubber
Major lipid classes[c]			
WE	39.0	(24.2–62.6)	n.d.[d]
TAG	61.0	(37.4–75.8)	100.0
Saturated and unsaturated fatty acids and alcohols[e]			
Saturated	94.6	(88.5–97.4)	24.9
Monounsaturated	3.9	(1.5–8.2)	28.3
Polyunsaturated	1.0	(0.0–3.3)	46.8
Typology of fatty acids and alcohols[f]			
Linear	13.8	(7.8–28.4)	81.7
Isobranched	28.4	(23.6–33.1)	6.7
Isovaleric[g]	57.9	(48.0–62.5)	11.7

[a]Errors in the integrated areas are less than 3%.
[b]Upper and lower measured values.
[c]Calculated from C-10 and C-11 signals in Figure 2a.
[d]Not detected.
[e]The monounsaturated and the polyunsaturated fatty acids and alcohols contents were measured from a deconvolution procedure applied to the olefinic region and normalized to the area of the carbonyl signals.
[f]The contents of isovaleric, isobranched, and linear fatty acids and alcohols were measured from normalized areas of C-12 and C-13, C-14, C-28 signals in Figure 2a, and areas of C-22, C-23, C-36, and C-37 signals in Figure 3a.
[g]Present only as fatty acid.

HMBC spectrum is shown in Figure 5. Here, two bonds apart, correlations between the carbonyl carbons and the protons of the methylene groups of isovalerate and of the remaining acyl groups are observed (cross-peaks between C-1, C-2, C-3 and H-10, H-8, H-9, respectively). These results, together with the observed correlations between C-1, C-2, C-3 and H-5, H-2, H-3 and H-4 signals, respectively (not shown in Fig. 5), permitted to fully assign signals in the carbonyl region of the melon raw oil ^{13}C spectrum.

Chemical shift attributions of ^{13}C NMR spectra of intact blubber tissue and the corresponding oil are reported in Table 3. From the analysis of the HSQC and HMBC spectra (spectra not shown) the following attributions in the carbonyl region were made: C-1 and C-2 signals were ascribed to carboxyl carbons of acyl groups other than isovalerate and DHA, in the *sn*-1,3 and *sn*-2 positions of TAG, respectively, while C-3 corresponds to the carboxyl carbons of isovalerate and DHA in the *sn*-1,3 positions of TAG. Moreover, the intense C-6 and C-8 signals in the olefinic region originate from the olefinic carbons in Δ9 monounsaturated acyl groups, the remaining signals were attributed to PUFA, DHA, and EPA acyl groups in TAG. Interestingly, in the glyceryl region an additional signal (C-21) attributed to the methylene carbon of glycerol esterified with isovalerate appears besides the characteristic resonances of glycerol methine (C-19) and methylene carbons (C-20). It is worth noting that the *sn*-1,3 positions of isovalerate in TAG in both blubber and melon was previously observed in odontocete blubber and acoustic oils (10).

Information on the lipid composition of melon and blubber tissues was obtained by the analysis of the integrated areas in the corresponding ^{13}C NMR spectra. The relative percentages of the different classes of fatty components are reported in Table 4. As far as the melon samples are concerned, a high variability of all the lipid components is observed. Furthermore, by comparing data from melon and blubber tissues, significant differences occur; indeed, blubber does not contain WE and exhibits more unsaturated lipids and less isobranched acyl groups than the melon. These results are in good agreement with previous data obtained by methods that involved preliminary solvent extraction of lipids from the tissues (2,3,6,21).

^{13}C spin-lattice relaxation. Dynamics of lipid components in melon tissue, its undiluted oil, and blubber tissue of the same individual were investigated by measuring the ^{13}C spin-lattice relaxation times, T_1 (Table 5). It is worth reminding that the relaxation times of the ^{13}C nucleus are dominated by the dipolar interaction with the covalently attached protons and give specific information on the mobility of the molecular groups (20). Generally, taking into account the number of directly attached protons, larger T_1 values indicate a higher mobility.

In Table 5 it can be observed that T_1 values measured in melon tissue and in oil are very similar, even if differences occur for the carboxyl carbons in TAG and WE. These results indicate that the lipids arranged in the melon tissue and in the oil exhibit a similar dynamic behavior, thus suggesting that the lipid molecules in the tissutal framework experience an overall liquid-like microenvironment with no significant restriction in their segmental mobility.

TABLE 5
^{13}C Spin-Lattice Relaxation Times T_1 (s), in Melon Tissue, the Corresponding Undiluted Raw Oil, and Blubber Tissue from *Stenella*

Compound	Carbon	T_1 (s)[a]		
		Melon tissue	Melon oil	Blubber tissue
Glycerol backbone TAG	-CH-OOC-	0.22	0.21	0.19
Glycerol backbone TAG	-CH_2-OOC-	0.14	0.13	0.12
TAG *sn*-2	-CH_2-OOC-CH_2-	3.55	1.93, 4.53	1.89
TAG *sn*-1,3	-CH_2-OOC-CH_2-	2.91	2.61	1.89
All a.g.[b]	-CH_2-OOC-CH_2-	0.27	0.25, 0.30	0.26
All a.g.	CH_2-OOC-CH_2-CH_2-	0.36	0.32	0.33
All a.g.	-$(CH_2)_n$-	0.40–0.46	0.48–0.35	0.46
Linear a.g.	ω3	0.94	0.87, 1.87	0.90
Linear a.g.	-CH_3	2.70	2.69	2.67
WE	-CH_2-OOC-CH_2-	2.33	1.79	n.d.[c]
Fatty alcohols in WE	-CH_2-OOC-	0.44	0.41	n.d.
Fatty alcohols in WE	-CH_2- CH_2-OOC-	0.52	0.45	n.d.
Isovaleric acid in WE	-COO-CH_2-CH-$(CH_3)_2$	0.89	0.90	n.d.
Isovaleric acid in TAG	-OOC-CH_2-CH-$(CH_3)_2$	0.56	0.53	0.45
Isobranched a.g.	ω3	0.77	0.71	0.66
Isovaleric a.g.	-CH_2-CH-$(CH_3)_2$	1.35	1.20	n.d.
MUFA	-CH=CH	0.63	0.58, 0.55	0.57

[a]Estimated errors within 5%.
[b]a.g. = Acyl groups.
[c]Not detected.

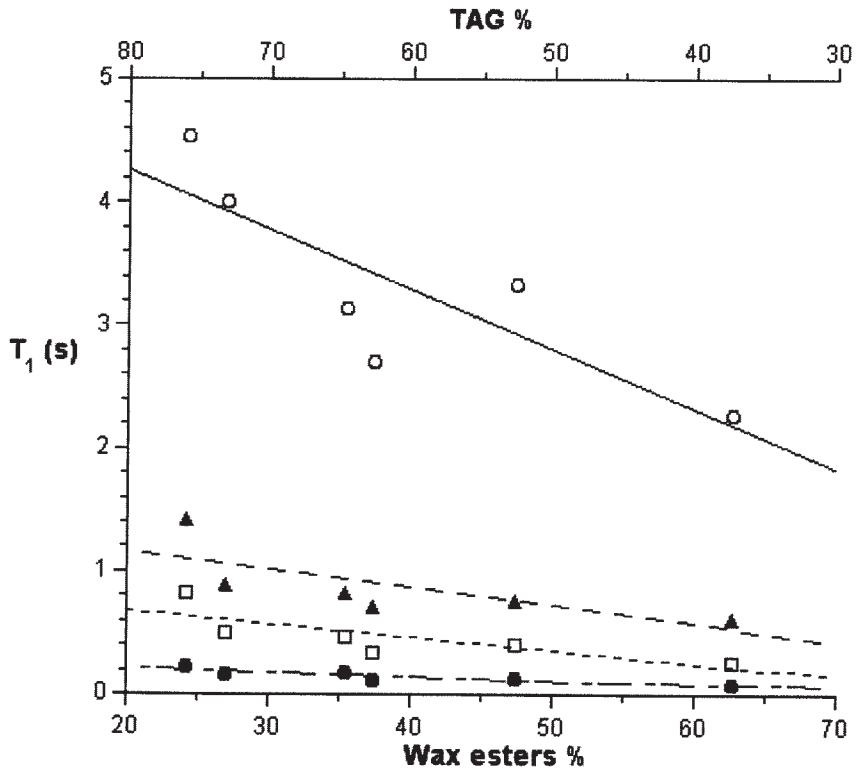

FIG. 6. ^{13}C NMR spin-lattice relaxation (T_1) vs. lipid composition for six samples of melon intact tissue of *Stenella*. The WE and TAG contents were obtained by the semiquantitative analysis of the ^{13}C NMR spectra. Symbols refer to signals in Figure 2: ○, signal 28 (-CH_3 in linear acyl groups); ▲, signal 14 (ω3 in isobranched f.a); □, signal 10 (-CH_2-OOC- in WE); ●, signal 11 (-CH_2-OOC- in TAG).

A comparison between the relaxation data of lipids in the melon and blubber tissues shows that a slight but systematic decrease of T_1 values occurs in the latter. This finding points to slower motions of fatty molecules in blubber than in melon tissue and may be ascribed to differences in the composition, although lipids experience a liquid-like environment also in blubber tissue.

To study the dependence of relaxation on the lipid composition in the tissutal framework, the ^{13}C spin-lattice relaxation times were measured in the six samples of melon intact tissue. In Figure 6 the T_1 values of selected well-resolved signals are reported as a function of WE and TAG relative contents. It can be seen that T_1 values decrease linearly as a function of the WE content increase, with a more pronounced slope for the -CH_3 functional groups. No linear dependence was observed for the other fatty components here examined. Therefore, in the light of these results it can be asserted that a more restricted mobility of lipids is induced by the increase of WE molecular components. Previous studies based on acoustic techniques demonstrated that also the sound properties of mixtures of lipids are influenced by the WE content, the increase of which appeared to decrease the ultrasonic velocity (7,8). The dependence of dynamics and sound properties of these acoustic lipids on the WE content suggests that this molecular component plays an important role in determining the heterogeneous three-dimensional map of lipid composition and sound velocity that makes the melon of odontocetes act as an acoustic lens.

In conclusion, this investigation demonstrates that NMR is a powerful tool for the study of composition and dynamics of the lipid matrices in intact tissues. Future works will be addressed to elucidate possible correlations between composition, dynamics, and acoustic properties in lipid matrices, thus contributing to shed light on the mechanism of the echolocation system in dolphins.

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Fatty Acid Composition of Muscle Fat and Enzymes of Storage Lipid Synthesis in Whole Muscle from Beef Cattle

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ABSTRACT: Enhanced intramuscular fat content (i.e., marbling) in beef is a desirable trait, which can result in increased product value. This study was undertaken with the aim of revealing biochemical factors associated with the marbling trait in beef cattle. Samples of *longissimus lumborum* (LL) and *pars costalis diaphragmatis* (PCD) were taken from a group of intact crossbred males and females at slaughter, lipids extracted, and the resulting FAME examined for relationships with marbling fat deposition. For LL, significant associations were found between degree of marbling and myristic (14:0, $r = 0.55$, $P < 0.01$), palmitic (16:0, $r = 0.80$, $P < 0.001$), stearic (18:0, $r = –0.58$, $P < 0.01$), and oleic (18:1c-9, $r = 0.79$, $P < 0.001$) acids. For PCD, significant relationships were found between marbling and palmitic ($r = 0.71$, $P < 0.001$) and oleic ($r = 0.74$, $P < 0.001$) acids. Microsomal fractions prepared from PCD muscle were assayed for diacylglycerol acyltransferase (DGAT), lysophosphatidic acid acyltransferase (LPAAT), and phosphatidic acid phosphatase-1 (PAP-1) activity, and the results examined for relationships with degree of intramuscular fat deposition. None of the enzyme activities from PCD displayed an association with marbling fat content, but DGAT specific activity showed significant positive associations with LPAAT ($r = 0.54$, $P < 0.01$), total PAP ($r = 0.66$, $P < 0.001$), and PAP-1 ($r = 0.63$, $P < 0.01$) specific activities. The results on FA compositions of whole muscle tissues provide insight into possible enzyme action associated with the production of specific FA. The increased proportion of oleic acid associated with enhanced lipid content of whole muscle is noteworthy given the known health benefits of this FA.

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The production of beef carcasses with high degrees of intramuscular fat deposition (i.e., marbling) is best achieved by the identification and selection of cattle with the genetic potential to efficiently deposit marbling fat, which contributes to beef flavor, texture, and quality grade (1). It is thought that selection for these animals might reduce the feeding period required to attain a high degree of marbling, while also having the effect of reducing fat accumulation in depots that impact the lean meat yield of the carcass. To achieve this goal, cow/calf producers require the information and tools to select herd sires and dams that will pass on the genetic predisposition for marbling to calves. Marbling is a heritable trait (2). Selection progress is most rapid when suitable herd animals can be identified at a relatively young age. The use of biochemical or genetic markers from samples of tissue greatly increases the rate of selection progress, especially if the marker(s) have predictive value early in the animal's life. The identification of suitable genetic or biochemical markers that can be applied in marker-assisted selection protocols will enable the beef industry to respond more efficiently and effectively to the market-driven demand for well-marbled beef.

Previous studies examining the possible relationship between marbling and the activity of fat-metabolizing enzymes and the FA composition of fat have been conducted using Japanese black hybrid cattle (3–5), which are known for their enhanced propensity to marble (6). These early studies used the readily accessible *pars costalis diaphragmatis* (PCD) (skirt muscle of diaphragm) as a source of enzymes rather than ribeye muscle (i.e., the *longissimus*), which was not accessible in a commercial abattoir situation until after extensive chilling of the carcass. Positive associations found previously in three separate studies (correlation coefficients, r, from 0.47 to 0.51, $P < 0.05$) between the fat content of the skirt muscle and ribeye suggested that the skirt muscle could serve as an appropriate source of stable enzyme activity (3–5). At least three biochemical properties associated with intramuscular fat content at maturity in Japanese black hybrid cattle have been identified (3–5). Two of these components are enzyme activities. Diacylglycerol acyltransferase (EC 2.3.1.20, DGAT) catalyzes the final step in the *sn*-glycerol-3-phosphate pathway leading to TAG, whereas hormone-sensitive lipase (EC 3.1.1.79, HSL) catalyzes the first step in the degradation of fat. DGAT activity

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Abbreviations: DGAT, diacylglycerol acyltransferase; HIP, hexane/isopropanol; HSL, hormone-sensitive lipase; LL, *longissimus lumborum*; LPAAT, lysophosphatidic acid acyltransferase; NEM, *N*-ethylmaleimide; PAP, phosphatidic acid phosphatase; PAP-2, phosphate phosphohydrolase; PCD, *pars costalis diaphragmatis*; SM, *semimembranosus*; TEL, total extracted lipid.

from intramuscular fat tissue appeared to decrease with increasing whole muscle fat content (3) whereas HSL activity in whole muscle increased as the whole muscle fat content increased (5). HSL activity of whole skirt muscle is positively associated with the fat content of both skirt and ribeye muscle. Myristic acid (14:0), although a minor constituent of fat, displayed a positive association with whole muscle fat content for both the skirt muscle and ribeye from Japanese black hybrid cattle (4).

The current study focused on the evaluation of FA composition of *longissimus lumborum* (LL) and PCD muscles as it relates to fat content of the muscles. Activities of DGAT, lysophosphatidic acid acyltransferase (LPAAT), and phosphatidic acid phosphatase (PAP) from PCD microsomes, key enzymes associated with intramuscular fat formation in bovine muscle, were also determined and evaluated with respect to possible associations with PCD lipid content.

EXPERIMENTAL PROCEDURES

Maintenance of cattle. The cattle in this study were from the offspring (F_1) generation of the Canadian Beef Cattle Reference Herd, established at the University of Saskatchewan to study the genetic basis of live animal, carcass, and meat quality traits (7,8). The herd consisted of a parental generation composed of Angus, Belgian Blue, Charolais, Horned Hereford, Polled Hereford, Limousin, and Simmental breeds, which were used to develop an offspring generation of crossbred families. The F_1 animals thus represented a population of 17 different types of crossbred cattle, representing a wide range of genetic diversity that was ideally suited to the further study of biochemical markers for the marbling trait. The cattle in this study included intact males and females and were cared for according to guidelines set by the Canadian Council on Animal Care (9).

Animals from the F_1 generation of the herd were fed in a manner consistent with typical western Canadian beef production. Calves were weaned between 150 and 180 d of age and, after an initial period (272 ± 12 d) of background feeding, animals were placed on a finishing diet (89 ± 8 d) prior to slaughter. The initial diet, formulated for 11.8 megajoules (MJ) digestible energy per kilogram dry matter and 12.5% (all diet percentages w/w) crude protein, contained 41% barley silage, 33.5% brome hay, 15% barley grain, 8.8% canola meal, 0.5% tallow, 0.5% $CaCO_3$, 0.5% mineral supplement, 0.1% vitamin supplement, and 0.1% salt. The finishing diet, formulated for 14.9 MJ digestible energy (kg dry matter)$^{-1}$ and 12.5% crude protein, comprised 78.8% barley grain, 11% barley silage, 4.9% canola meal, 3.6% tallow, 0.9% $CaCO_3$, 0.4% salt, and 0.4% vitamin supplement.

Procurement and storage of samples. Animals were stunned by pneumatic captive bolt, exsanguinated, and dressed in a simulated commercial manner. Immediately following exsanguination a small cut was made in the hide of the animal, immediately posterior to the last rib, approximately 12 cm off the midline of the back. A sharpened cork borer (37 mm diameter) was inserted perpendicular to the hide, to a depth of approximately 8 cm. The muscle core was removed, and the subcutaneous fat was trimmed from the core prior to freezing in liquid nitrogen. Following removal of the LL core, the right *semimembranosus* (SM) muscle was accessed along the rear inside of the right leg, approximately 10 cm distal to the aitch bone. A cut was made through the hide, and through the overlying *gracillis* muscle. The core was then removed from the central portion of the SM, approximately one-third of the way down the length of the muscle from the proximal end with a 37-mm sharpened cork borer. The standardization of the location of the cores was confirmed following post-mortem dissection of the muscles.

Approximately 10 g of PCD muscle was sampled immediately following gutting, from the medial crus of the diaphragm at the level of the 10th thoracic vertebrae from within the abdominal cavity. All muscle samples were immediately frozen in liquid nitrogen and held frozen at –80°C until processed for lipid extraction and microsome preparation. After 24 h, the left sides of each carcass were fully evaluated for grade characteristics, including the assessment of American Meat Science Association (AMSA) marbling scores by an experienced meat quality biologist, who has over 20 yr of experience in carcass assessment and who is also a certified Canadian Beef Grading Agency grader. A photographic reference scale describing the various levels of marbling was used (Official USDA Marbling Photographs, National Livestock and Meat Board, Chicago, IL).

Lipid analysis. Following grading, samples of LL and SM muscles were collected from the left sides of all carcasses (n = 126). Following a week of vacuum-packaged storage at 2°C, muscle samples were ground three times [Butcher Boy Meat Grinder Model TCA22 with a 1/8-in. grind plate (Lasar Manufacturing Co., Los Angeles, CA)]. One hundred grams of the grind were weighed into stainless steel beakers and placed in a gravity convection drying oven (Model 1370FM; VWR Scientific, Mississauga, Ontario, Canada) at 105°C for 24 h. Beakers were removed from the oven, final masses recorded, and moisture losses calculated. The dried samples were pulverized to a fine grind and crude fat content determined by petroleum ether extraction (Tecator Soxtec System HT-1043; Tecator Ltd., Hoganas, Sweden).

A subset of 22 animals, representing the range of marbling from all available samples, was chosen for further FA and muscle enzyme activity analysis. These tissues were chosen solely on the basis of carcass marbling score and represented the following ranges: marbling score 100–290 (“devoid” and “practically devoid” of marbling), n = 5; marbling score 300–390 (“traces”), n = 3; marbling score 400–490 (“slight”), n = 4; marbling score 500–590 (“small”), n = 6; and marbling score 600–690 (“modest”), n = 4. Hexane/isopropanol (HIP) lipid extraction, methylation, and FA analysis by GC (4) were performed on PCD and LL muscles from this subset. Relative proportions, calculated as mol%, of all known FA were then statistically compared with measures of intramuscular fat (i.e., marbling score and lipid extraction data from both muscles) using the Pearson product-moment correlation method

(10) to identify FA exhibiting associations with the marbling trait.

Enzyme activity analysis. Microsomal fractions were prepared by differential centrifugation as described previously (3). Microsomes were prepared from the PCD muscle from the animals (n = 22, same subset as above). Microsomal protein content was determined using the Bio-Rad protein microassay (Hercules, CA) with BSA as the standard. All protein and enzyme assays were performed in triplicate. DGAT assays were performed as previously reported by Middleton *et al*. (3). PAP assays were conducted in the presence and absence of 0.5 mM *N*-ethylmaleimide (NEM) in order to distinguish between PAP-1 and PAP-2 activities (11,12). LPAAT assays were performed on microsomal fractions from the PCD muscle based on the assay published by Schlossman and Bell (13). As with the FAME analyses, enzyme specific activities were analyzed with respect to measures of intramuscular fat content for possible associations between enzyme activity and marbling fat deposition.

RESULTS AND DISCUSSION

While not necessarily conducive to the deposition of extensive amounts of intramuscular fat, the animals in this study were killed at a constant age because this allowed for a meaningful assessment of the variation in slaughter traits without having a measured trait as the factor that determined end point (e.g., through body weight or live ultrasound measurement). This strategy, as well as the inclusion of bulls (as opposed to steers) in the study, led to somewhat lower marbling scores in the experimental animals than those in typical North American commercial slaughters. The range in intramuscular fat deposition, as measured through both solvent extraction and marbling score, is shown in Figure 1.

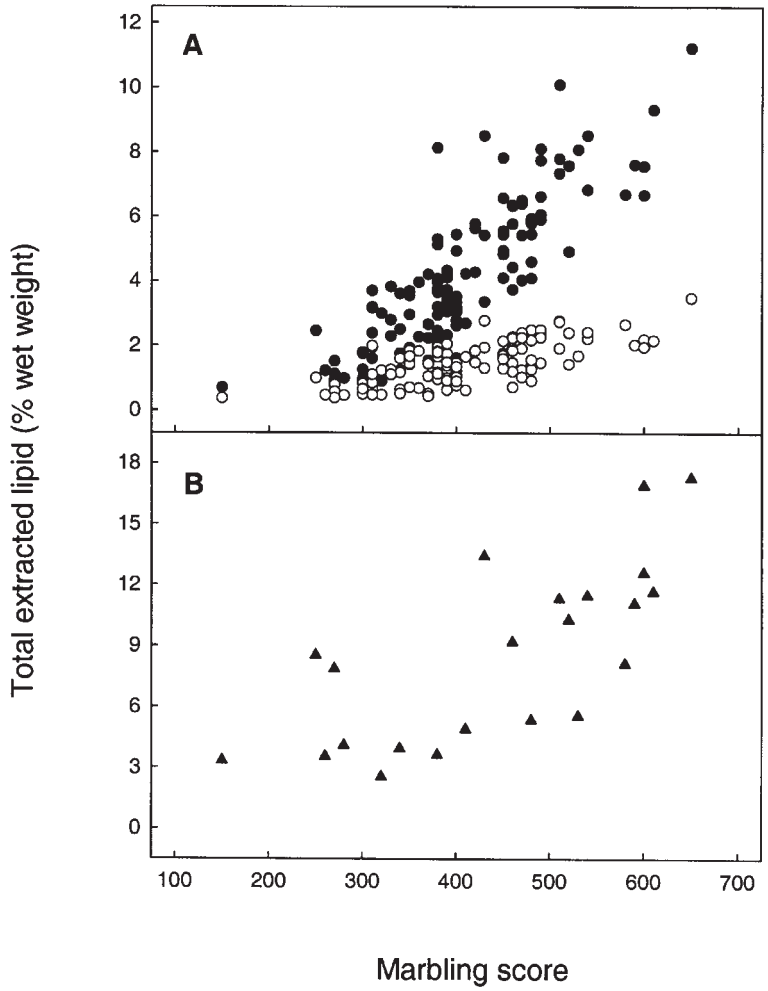

FIG. 1. Relationship between carcass marbling score and the amount of fat extracted from several bovine muscles. Panel A: Data from *longissimus lumborum* (●) and *semimembranosus* (○) muscles (n = 126). Panel B: Data from *pars costalis diaphragmatis* muscle (▲) from the subset of animals used for analyses of FA compositions and enzyme activities (n = 22). Pearson correlation coefficients (r) for the relationship between the lipid extract data and marbling score were 0.83 ($P < 0.0001$) for *longissimus lumborum,* 0.70 ($P < 0.0001$) for *semimembranosus,* and 0.73 ($P < 0.001$) for *pars costalis diaphragmatis.*

TABLE 1
FA Composition[a] of Extracted Lipid and Muscle Lipid Content of Bovine PCD (*pars costalis diaphragmtis*) and LL (*longissimus lumborum*) Muscle Samples (*n* = 22)

	Mol%	
FAME	PCD	LL
14:0	1.79 ± 0.136	1.98 ± 0.211
14:1 *cis*-9	0.33[a] ± 0.043	0.50[b] ± 0.059
15:0	0.43 ± 0.015	0.40 ± 0.018
15:1 *cis*-10	1.39[c] ± 0.175	2.80[d] ± 0.431
16:0	22.59[c] ± 0.452	25.32[d] ± 0.721
16:1 *cis*-9	1.89[c] ± 0.062	3.20[d] ± 0.208
16:1 *trans*-9	0.53[c] ± 0.011	0.38[d] ± 0.024
17:0	1.55[c] ± 0.050	1.07[d] ± 0.029
18:0	22.16[c] ± 1.116	14.56[d] ± 0.352
18:1 *cis*-9	37.48 ± 1.116	36.29 ± 1.506
18:1 *cis*-11	1.75 ± 0.047	1.88 ± 0.075
18:2 *trans*-9,*trans*-12	0.19 ± 0.029	0.24 ± 0.026
18:2 *cis*-9,*cis*-12	5.76 ± 0.705	7.50 ± 1.239
18:3 *cis*-9,*cis*-12,*cis*-15	0.45[a] ± 0.030	0.66[b] ± 0.090
20:3 *cis*-11,*cis*-14,*cis*-17	0.26[c] ± 0.034	0.53[d] ± 0.080
20:3 *cis*-8,*cis*-11,*cis*-14	1.21[a] ± 0.140	2.01[b] ± 0.306
24:0	0.24[c] ± 0.034	0.68[d] ± 0.089
Lipid (% wet wt.)	8.48[c] ± 0.942	3.06[d] ± 0.451

[a]Values are mean ± SE. Means with the following pairs of superscripts within the same row differ significantly (SAS General Linear Model): [a,b]($P < 0.05$), [c,d]($P < 0.01$).

Association of total extracted fat with marbling score. Previous investigations of the relationships between muscle fat content and DGAT from PCD intramuscular adipose tissue (3), PCD FA composition (4), HSL activity from PCD muscle (5), and plasma leptin concentration (14) were conducted using Japanese black hybrid cattle (3–5,14). The current study has extended these investigations to examine cattle breeds that are more typically found in North American herds. Highly significant relationships between total fat content determined through Soxhlet extraction and the USDA marbling score were found for the two muscles studied from the F_1 herd (Fig. 1A). For LL and SM muscle, the association (r) between extracted lipid and marbling score was 0.83 ($P < 0.0001$) and 0.70 ($P < 0.0001$), respectively, and the relationship was stronger when lipid extraction data from the muscles were compared ($r = 0.87$, $P < 0.0001$). HIP extraction of lipid samples from PCD muscles of a subset ($n = 22$) of the F_1 herd also revealed highly significant associations between muscle lipid content and marbling score ($r = 0.73$, $P < 0.0001$; Fig. 1B) as well as between lipid content of this muscle and that of LL ($r = 0.83$, $P < 0.0001$) and SM ($r = 0.85$, $P < 0.0001$). Collectively, these results show that marbling score accurately reflects the amount of fat content in various bovine muscles and that intramuscular fat deposition is related among muscles that exhibit marbling. These results are supported by previous reports, both from our laboratory (4) and elsewhere (15).

Association of relative proportions of myristic, palmitic, and oleic acids with muscle lipid content. FA analysis of PCD and LL muscle was conducted using HIP lipid extracts prepared from muscle samples of a subset ($n = 22$) of the F_1 herd. The carcasses from the animals in this subset displayed a wide range in marbling score (i.e., from 150 to 650). Average values (±SE) for FA composition of total extracted lipid (TEL) from the two muscles from the subset of the F_1 herd are presented in Table 1. In general, the FA composition of the TEL of the two muscles was similar. The proportion of palmitic acid (16:0), however, was higher in LL than in PCD muscle and, conversely, stearic acid (18:0) in PCD was considerably greater than in LL muscle. These combined results implicate greater 16:0–18:0 elongase activity in the PCD. In contrast, analyses of the FA composition of TEL as a function of muscle lipid content or marbling score revealed a number of strong associations. Significant positive associations were found between the relative proportions of myristic (14:0; $r = 0.55$, $P = 0.0078$), palmitic ($r = 0.80$, $P < 0.0001$), and oleic (18:1 *cis*-9; $r = 0.79$, $P < 0.0001$) acids in relation to the TEL content of LL muscle, whereas a significant negative association ($r = –0.58$, $P = 0.0047$) was found for 18:0 for this muscle (Fig. 2). Positive associations were noted for 16:0 ($r = 0.71$, $P = 0.0002$) and 18:1 *cis*-9 ($r = 0.74$, $P = 0.0001$) in relation to TEL content of PCD muscle.

Kazala *et al.* (4) reported a significant positive association between the relative proportion of 14:0 and both total acyl lipid and TAG extracted from both PCD and *longissimus* muscle from Japanese black hybrid cattle. This association also has been noted by other groups (16,17). The fact that this relationship was detected in breeds more representative of North American beef production supports this trait as being present in the *Bos taurus* species as a whole, and not just specific to the Japanese black breed. The positive associations observed between 16:0 and TEL content of both muscles sampled in the present study were also detected in a previous study of Japan-

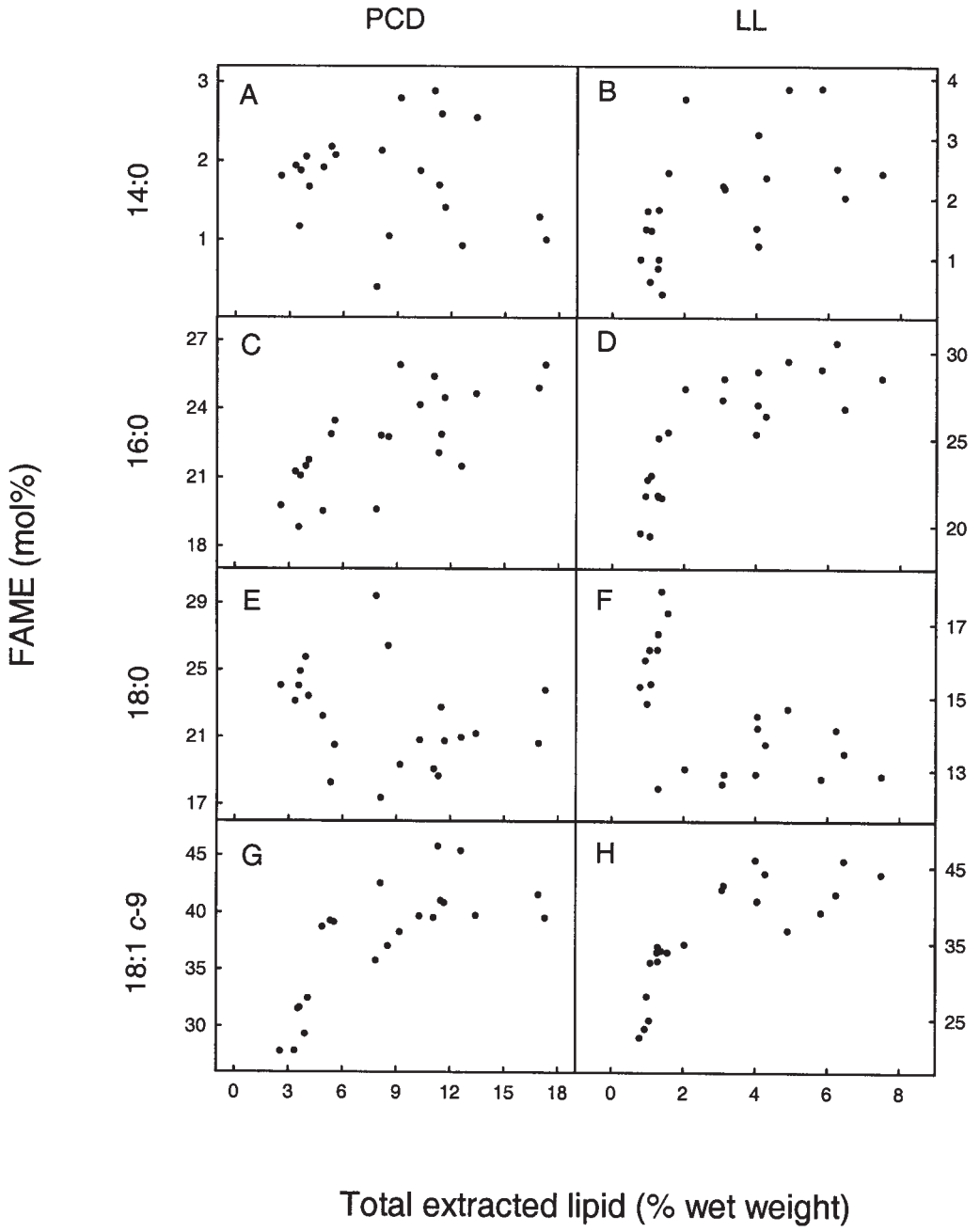

FIG. 2. Relationship between the amount of fat extracted from whole PCD (*pars costalis diaphragmatis*) and LL (*longissimus lumborum*) muscles and the relative content of several FAME in the extracted fat ($n = 22$). Correlation coefficients (r) for each panel and corresponding P values (in parentheses) are as follows: A, –0.12 (0.60); B, 0.55 (0.0078); C, 0.71 (0.0002); D, 0.80 (<0.0001); E, –0.29 (0.19); F, –0.58 (0.0047); G, 0.74 (0.0001); H, 0.79 (<0.0001).

ese black hybrid cattle (4). The association, however, was weaker ($r = 0.44$) for Japanese black hybrid cattle and only apparent in PCD muscle, not in *longissimus* muscle. It should be noted that, aside from breed differences between the present work and past studies involving Japanese black hybrid animals, the animals in previous studies (3–5) were slaughtered according to physical maturity measures (i.e., live weight and backfat thickness as determined through ultrasonography). Collectively, these differences would likely have a substantial effect on differences in intramuscular fat deposition, and this should be kept in mind when considering differences in the findings. Conversely, it can be argued that similar relationships found in both types of studies are likely to represent characteristics of the *Bos taurus* species, being present despite considerable differences in breed type and finishing regime.

In the present study, the correlations between relative proportions of both 14:0 and 16:0 and TEL content of LL muscle implicate the FA synthase complex as having a key role in gov-

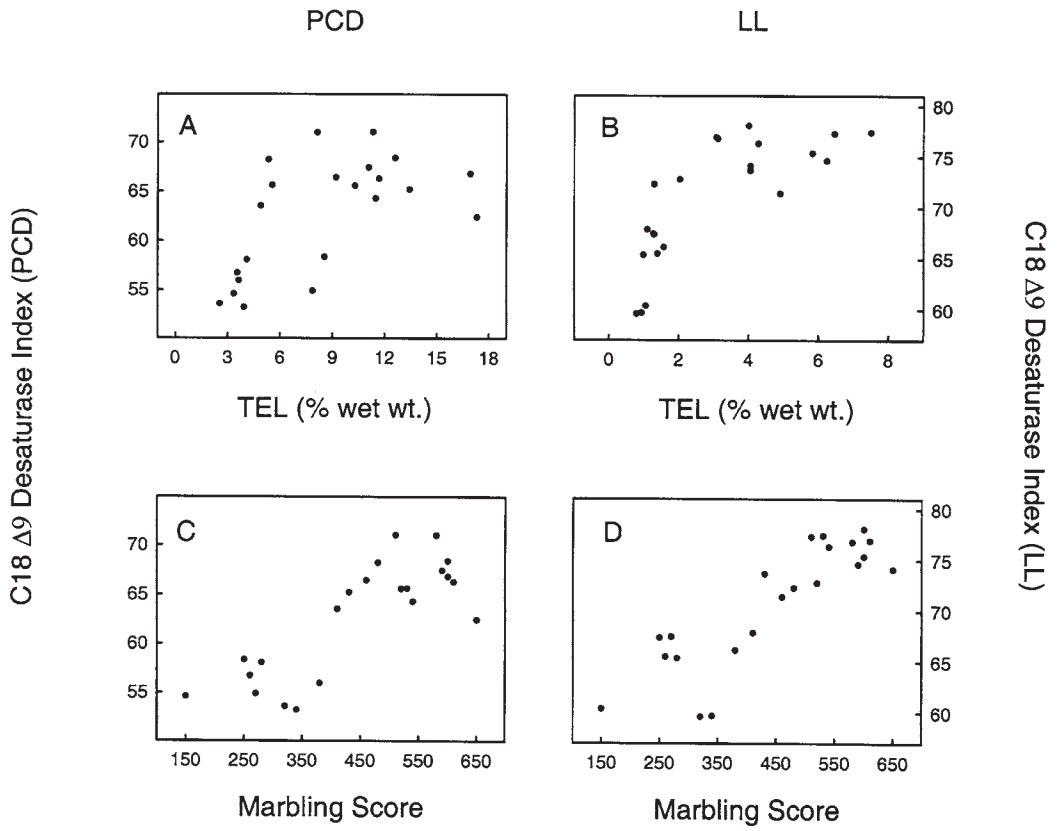

FIG. 3. Relationship between index of C18 Δ9 desaturase activity and two measures of muscle fat content. PCD, *pars costalis diaphragmatis*; LL, *longissimus lumborum*; TEL, total extracted lipid; C18 Δ9 desaturase index = [18:1 *cis*-9/(18:0 + 18:1 *cis*-9)]100. Correlation coefficients (*r*) for each panel and corresponding *P* values (in parentheses) are as follows: A, 0.60 (0.0034); B, 0.77 (<0.0001); C, 0.81 (<0.0001); D, 0.86 (<0.0001).

erning these associations. The typical end product of FA synthase action is 16:0, with 4- to 14-carbon products released in some mammalian tissues (18). The positive relationship presented for LL muscle suggests that the FA synthase complex may be somewhat more prone to 14:0 production in animals showing greater degrees of marbling. The increased proportions of 14:0 and 16:0 in TEL that occur with increased muscle fat content, however, might also be attributable to changes in acyltransferase selectivity. These enzymes are responsible for catalyzing the addition of fatty acyl moieties onto the glycerol backbone in the pathway leading to TAG (19,20). Factors such as substrate availability and/or possible allosteric modulation of one or more of these acyltransferases may have resulted in changes in selectivity such that 14:0 and 16:0 were more preferentially incorporated into TAG in animals with higher muscle fat content.

Of the FA examined, the proportion of 18:1 *cis*-9 in the TEL of both muscles showed the greatest increase as a function of muscle lipid content. Skelley *et al*. (17) reported a significant positive relationship between the relative content of 18:1 *cis*-9 in lipid extract of *longissimus* muscle and marbling score. Kazala *et al*. (4) reported a similar association between this FA and TEL content of PCD muscle from Japanese black hybrid cattle, although the relationship was not statistically significant ($0.05 < P < 0.1$). The relative increase of 18:1 *cis*-9 observed as TEL content increased might be due to increases in 18:0 Δ9 desaturase activity. The index of C18 desaturase activity as a function of TEL of PCD and LL muscle is shown in Figures 3A and 3B, respectively. The relationship between the index of C18 desaturation for each muscle in relation to marbling score at the grading site is depicted in Figures 3C and 3D. A highly significant positive association was found in each case. Thus, it might be useful to evaluate Δ9 stearoyl-CoA desaturase activity in relation to muscle lipid content. The selectivities of acyltransferases, which catalyze the transfer of fatty acyl moieties from the acyl-CoA pool onto glycerolipids, might also contribute to the increased proportion of 18:1 *cis*-9 in total acyl lipid of TEL as muscle lipid content increases.

Although the underlying reason(s) for the positive association between 18:1 *cis*-9 and the amount of marbling fat remain unclear, these results are relevant from a dietary and/or marketing perspective. Oleic acid, the predominant FA moiety in such "heart-healthy" vegetable oils as canola oil and olive oil, reduces both plasma total cholesterol and total LDL cholesterol in humans (21,22; reviewed in 23). Additionally, taste panel evaluations of tenderness, juiciness, and flavor of cooked *longissimus* report a more desirable flavor when increased amounts of 18:1 *cis*-9 are present (16); and similar trends be-

TABLE 2
Activities of Enzymes Involved in Lipid Metabolism, from Subcellular Preparations of Bovine *Pars Costalis Diaphragmitis* Muscle Samples (n = 22)

Enzyme	Specific activity	SE	Activity FW	SE
LPAAT[a]	3.03	0.717	2.23	0.133
PAP (total)[b]	2.21	0.224	0.57	0.059
PAP-1[c]	0.93	0.233	0.25	0.060
DGAT[d]	0.13	0.031	0.10	0.006

[a]LPAAT, lysophosphatidate acyltransferase; specific activity, nmol phosphatidic acid min^{-1} mg $protein^{-1}$; activity FW, nmol phosphatidic acid min^{-1} g fresh $weight^{-1}$.
[b]PAP (total), total phosphatidate phosphatase; specific activity, nmol P_i min^{-1} mg $protein^{-1}$; activity FW, nmol P_i min^{-1} g fresh $weight^{-1}$.
[c]PAP-1, phosphatidate phosphatase-1; specific activity, nmol P_i min^{-1} mg $protein^{-1}$; activity FW, nmol P_i min^{-1} g fresh $weight^{-1}$.
[d]DGAT, diacylglycerol acyltransferase; specific activity, nmol TAG min^{-1} mg $protein^{-1}$; activity FW, nmol TAG min^{-1} g fresh $weight^{-1}$.

tween level of marbling, flavor intensity, and 18:1 *cis*-9 content were found in a more recent study by Camfield *et al*. (24) of crossbred feedlot steers, although their findings were statistically significant only between the extremes of finishing diet regimen and resulting marbling fat deposition. The results presented here show that as marbling fat content increases, so does the relative proportion of 18:1 *cis*-9 contained therein, resulting in better-tasting beef with the beneficial health effects associated with increased proportions of this FA.

Activities of lipogenic enzymes in muscle tissue samples. Analyses of enzyme activities involved in TAG assembly were conducted using microsomes prepared from PCD muscle of the same subset of animals used for FA analysis. Investigations of the relationship between enzyme activity and muscle lipid content in Japanese black hybrid cattle have been conducted using this muscle (3,5). Mean values for activities of three enzymes involved in TAG synthesis are shown in Table 2. Activities are shown on both a specific activity and activity per g fresh weight basis. In general, DGAT displayed the lowest activity of all three enzymes in the pathway. This is supported by a previous report, using the DGAT inhibitor 2-bromooctanoate on studies of rat hepatocytes, that showed that DGAT may have a rate-limiting role in the production of TAG (25).

Both total PAP activity and PAP activity specifically involved in glycerolipid synthesis (PAP-1) were measured. PAP-1 activity is responsible for generating the DAG required by DGAT to form TAG (19), whereas PAP-2, also referred to as phosphate phosphohydrolase and located in the plasma membrane, is involved in cellular signaling processes (26). Assays conducted in the presence of NEM resulted in the inhibition of PAP-2 activity involved in signal transduction, thereby allowing PAP-1 activity to be determined by subtraction of PAP-2 from total PAP activity (11). The relationships of the specific activities of the enzymes to each other are presented in Table 3. DGAT activity showed significant positive associations with LPAAT and both measures of PAP activity. The data suggest that the two acyltransferase activities were coordinated and that DGAT activity was coordinated with PAP-1 activity in the various samples of PCD muscle. None of these enzyme activities, however, was significantly associated with muscle fat content or marbling score (i.e., $P \geq 0.05$). In contrast, investigations of lipid synthesis in Japanese black hybrid cattle showed a significant negative association between microsomal DGAT specific activity of intramuscular fat and lipid content of the PCD (3). In that study, however, intramuscular fat was physically dissected from the surrounding muscle, and microsomal fractions were prepared from the muscle-free intramuscular fat. Also, the study used Japanese black hybrid cattle, which are not representative of the more typical Canadian breeds used in the present study. The generally lower degree of marbling in the present group of cattle, compared with Japanese black hybrid cattle, made it extremely difficult to dissect out sufficient amounts of intramuscular fat from the whole muscle for biochemical evaluation.

Summary and implications for the beef industry. The cattle used in the current study displayed a highly significant association between muscle fat content of LL and SM muscles and marbling score at the grading site. Significant positive associations between 14:0 or 16:0 FA moieties from bovine muscle lipid extracts (PCD and LL) and the amount of fat present in the same muscles implicate the activity of FA synthase and/or acyltransferase selectivity as having a role in determining the FA composition of marbling fat. The significant positive association between 18:1 *cis*-9 moieties from whole bovine muscle lipid extracts (PCD and LL) and the amount of muscle fat implicate the activity of Δ9 stearoyl-CoA desaturase and/or acyltransferase selectivity as having a role in determining the proportion of 18:1 *cis*-9 content in muscle fat. A reasonable starting point for investigating the possible involvement of specific acyltransferases contributing to the FA composition of bovine muscle fat would involve a study of the positional distribution of FA as a function of muscle lipid content. Thus, changes in acylation due to acyltransferase selectivity could be linked to specific positions on the glycerol backbone of TAG. None of the TAG bioassembly enzymes of the PCD muscle studied showed a significant association with marbling fat deposition. Microsomal DGAT and LPAAT activities, however, appeared to be coordinated in the various samples of PCD muscle.

The significant associations detected between 18:1 *cis*-9 from lipid extracts and extent of marbling should be verified in

TABLE 3
Relationship[a] of the Specific Activities of Several Enzymes Involved in Lipid Metabolism to Each Other

	DGAT[b]	PA phosphatase-1[c]	PA phosphatase (total)[d]
LPAAT	0.54[y]	0.09	0.16
PAP (total)	0.66[z]	0.91[z]	—
PAP-1	0.63[y]	—	—

[a]Enzymes from subcellular preparations of *pars costalis diaphragmatis* muscle samples were assayed ($n = 22$). Pearson correlation coefficients are shown. [y]$P < 0.01$. [z]$P < 0.001$.
[b]Diacylglycerol acyltransferase; nmol triacylglycerol min^{-1} mg $protein^{-1}$.
[c]Phosphatidate phosphatase-1; nmol P_i min^{-1} mg $protein^{-1}$.
[d]Total phosphatidate phosphatase; nmol P_i min^{-1} mg $protein^{-1}$.

more well-marbled carcasses (i.e., with more representation from modest, moderate, and abundant categories) and, if they hold, exploited with respect to marketing well-marbled beef. This particular monounsaturated FA is the major FA found in canola and olive oils and has been shown to help reduce plasma total cholesterol and total LDL cholesterol in humans. The results presented here show that as the amount of marbling fat increases, so does the relative proportion of this beneficial FA. The activities of TAG bioassembly enzymes should be examined over the development of beef cattle to better understand the relationship between these enzyme activities and marbling fat. Biochemical differences between animals with different propensities to marble might become apparent at earlier stages of development. Trends detected earlier in beef cattle development would be more useful from a marbling predictability standpoint.

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Bovine Muscle n-3 Fatty Acid Content Is Increased with Flaxseed Feeding

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ABSTRACT: We examined the ability of n-3 FA in flaxseed-supplemented rations to increase the n-3 FA content of bovine muscle. Two groups of animals were used in each of two separate trials: (i) Hereford steers supplemented (or not) with ground flaxseed (907 g/d) for 71 d, and (ii) Angus steers supplemented (or not) with ground flaxseed (454 g/d for 3 d followed by 907 g/d for 110 d). For the Hereford group, flaxseed-supplemented rations increased 18:3n-3 (4.0-fold), 20:5n-3 (1.4-fold), and 22:5n-3 (1.3-fold) mass as compared with the control, and increased total n-3 mass about 1.7-fold. When these data were expressed as mol%, the increase in 18:3n-3 was 3.3-fold and in 20:5n-3 was 1.3-fold in the phospholipid fraction, and 18:3n-3 was increased 4-fold in the neutral lipid fraction. For the Angus group, flaxseed ingestion increased masses and composition of n-3 FA similarly to that for the Herefords and doubled the total n-3 FA mass. The effect of cooking to a common degree of doneness on FA composition was determined using steaks from a third group of cattle, which were Angus steers. We demonstrated no adverse effects on FA composition by grilling steaks to an internal temperature of 64°C. Because n-3 FA may affect gene expression, we used quantitative real-time reverse transcriptase-polymerase chain reaction to quantify the effect of feeding flaxseed on heart-FA binding protein, peroxisome proliferator activated receptor γ (PPARγ) and α (PPARα) gene expression in the muscle tissue. PPARγ mRNA level was increased 2.7-fold in the flaxseed-fed Angus steers compared with the control. Thus, we demonstrate a significant increase in n-3 FA levels in bovine muscle from cattle fed rations supplemented with flaxseed and increased expression of genes that regulate lipid metabolism.

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Most people on a Western diet consume much lower levels of n-3 FA than the recommended daily amounts and much higher levels of n-6 FA than is ideal for good health (1). Few of these people routinely eat the fish that are good sources of 20:5n-3 and 22:6n-3. Further, there is increasing concern about contaminants such as mercury and dioxins that may limit the healthfulness of high fish intake (2,3). In the United States, the average annual per capita fish intake is only 7.4 kg (4), with only some of this intake of species containing high levels of n-3 FA (5). On average, Americans eat four times as much beef as fish (mean annual per capita intake is 30 kg), and studies have shown that cattle consuming forages or feeds containing n-3 FA can produce meat with higher levels of n-3 FA and lower levels of n-6 FA (6–8).

Consequently, n-3 FA-enriched beef has the potential to help beef-eating Americans increase their n-3 FA intake. For example, a person who consumes 227 g (8 oz) of n-3-enhanced beef containing 155 mg of n-3 FA (18:3n-3, 20:5n-3, 22:5n-3, and 22:6n-3) per 100 g (8) could consume as much as 352 mg/d of total n-3 FA. For individuals who consume little or no fish with high n-3 levels, flaxseed-fed n-3-enriched beef consumed along with other n-3 sources could provide a diet with adequate n-3 intake (9). A diet with adequate n-3 intake, but essentially fish-free, could include flaxseed, walnuts, other 18:3n-3 sources in baked products and cereals, flaxseed oil in salad dressings, and perhaps eventually genetically modified plant sources of EPA and DHA, as well as n-3-enhanced dairy products (10–12), pork (13,14), lamb (15–17), chicken, and chicken eggs (18,19).

There is small but growing interest in North America in growing and fattening cattle on forage diets that are obtained primarily from grazing instead of the traditional feeding of high-grain diets to penned cattle. This interest relates to several beliefs including that meat from forage-consuming animals is healthier because of its composition and quantity of FA (8,20–22). However, because of the short growing season for the northern Great Plains of North America it is difficult to fatten yearling cattle to slaughter stage while grazing grass or grass-legume pastures. To overcome this problem, we grazed cattle on whole standing corn for the last 2–4 mon of their lives. Even after a killing frost and loss of nutritious corn leaves, cattle can still obtain enough energy by consuming the corn ears to fatten quickly. Unfortunately, corn grain has low levels of 18:3n-3 and high levels of 18:2n-6, so enrichment of beef with n-3 FA under this scenario will be impossible unless they also ingest a concentrated n-3 FA source such as flaxseed.

In light of these concerns, we conducted two field trials to determine whether the n-3 FA levels of muscle could be elevated in cattle finished on corn with supplemental flaxseed. Additionally, because there are mixed reports in the literature with respect to the effect of cooking on n-3 FA levels in beef (22–25), we evaluated the effect of cooking on n-3 FA levels contained in our n-3-enriched beef.

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Abbreviations: CYC, cyclophilin; H-FABP, heart-FA binding protein; M-MLV, Moloney murine leukemia virus; PPAR α and -γ, peroxisome proliferator activated receptor α and -γ; RT-PCR, reverse transcriptase polymerase chain reaction.

EXPERIMENTAL PROCEDURES

Feeding regimen for Hereford cattle for muscle enrichment. Eleven Hereford steers (castrated males) that were about 17 mon old with mean body weight of 510 kg (SD = 17 kg) at the beginning of the study were individually fed mixtures of grain each morning for 71 d while they grazed standing corn together in one group. Three and a half months prior to the commencement of the study, all steers grazed the same perennial grass. Six (controls) were fed mixtures of corn and field peas, and five (treatment) were initially fed mixtures of peas and ground flaxseed (907 g/d) and later mixtures of corn, peas, and ground flaxseed (corn was added to increase the energy intake of both groups as the weather cooled considerably). All animals were given 40 IU/d of Vitamin E and 29 g/d of calcium carbonate with the grain supplement. All animals had equal access to standing corn for grazing, and we balanced the supplements for available energy. The ground flaxseed was treated before feeding with the lignosulfonate process (26). At the end of the 71-d period, a biopsy was taken from the *longissimus dorsi* muscle of each animal between the 12th and 13th ribs. The muscle was immediately frozen and stored at –20°C until analysis.

Feeding regimen for Angus cattle for muscle enrichment. Twenty Angus steers that were about 16 mon old and had a mean body weight of 414 kg (SD = 39 kg) at the start of the study were used. Ten steers were supplemented with flaxseed and 10 control steers were not supplemented with anything. All of these cattle grazed grass pasture from early June to mid-July, then they grazed a mixture of oats and peas during the second half of July. During August they grazed stands of immature proso millet, and during September and October they grazed whole standing corn. For the first half of September, corn plants were immature and green, but began to die after a killing frost in mid-September. Through October the steers selected primarily the ears off the plants thus consuming mainly grain and cob. Ground flaxseed (nontreated) supplementation began on July 15 and continued through October. For the first 3 d of the trial, each steer was offered only 454 g/d of ground flaxseed to allow them to adjust to the new grain and then received 907 g/d for the remainder of the trial (107 d). Ground flaxseed was offered and consumed each morning while the steers were on the fields described above. While the cattle grazed the oat–pea and corn stands, a small amount (typically 45 to 65 g) of molasses powder was added to the flaxseed to increase its desirability. The steers were slaughtered at the end of the flaxseed supplementation period and cross-sectional pieces of *longissimus dorsi* and minor associated muscles (i.e., ribeye steaks) were collected from each carcass between the 12th and 13th ribs. To approximate the treatment of steaks prepared for human consumption, these steaks were stored in a cooler for 15 d at 4°C (i.e., "aged") then stored frozen until analysis.

Feeding regimen for Angus cattle for steak grilling trial. Ten Angus steers that were about 18 mon old and had a mean body weight of 351 kg (SD = 33 kg) at the beginning of the study were used. They were grazed together on grass pastures for 5 mon before flaxseed feeding began. They were fed a mixture of ground nontreated flaxseed and grain for 132 d before they were slaughtered. Ground flaxseed was fed at a rate of only 454 g/d for the first 22 d for an adjustment phase followed by 907 g/d for the final 110 d. The grain was a mixture of triticale, oats, peas, and corn, and the amount of feed was increased as the weather became colder and the cattle became more adapted to high grain diets. All animals were given 40 IU/d of Vitamin E and 29 g/d of calcium carbonate with the grain supplement. Grass hay was available to the cattle *ad libitum* during the 132-d feeding period. At the end of the feeding period steers were slaughtered; half of the carcasses had a quality grade of "Choice" and the other half were graded "Select" by standards of the U.S. Department of Agriculture. The carcasses were stored in a cooler for 12 d at 2°C (aged), then ribeye steaks that were about 2.5 cm thick were collected from each carcass between the 12th and 13th ribs, and these were stored at –20°C until analysis.

Meat preparation and cooking. Frozen ribeye steaks from the Angus cattle were slowly thawed in a refrigerator. Core samples were taken from the *longissimus dorsi* muscle of these steaks prior to grilling and flash-frozen in liquid nitrogen to minimize changes in tissue FA composition. Steaks were grilled on a gas barbecue to an internal temperature of about 64°C (considered "medium" in the United States and a popular degree of doneness for beef steaks) and another sample was cored from the cooked muscle for analysis and then flash-frozen in liquid nitrogen. Frozen tissue was pulverized under liquid nitrogen temperatures and extracted as described below.

Tissue lipid extraction. Tissue cores were taken from visibly lean portions of the frozen steaks and immersed in liquid nitrogen. These samples were then pulverized under liquid nitrogen temperatures into a fine homogeneous powder. Lipids from the tissue powder were extracted using a single-phase extraction with *n*-hexane/2-propanol (3:2 vol/vol) (27). After centrifugation at 1,000 × *g* to pellet debris, the lipid-containing liquid phase was removed and stored under a nitrogen atmosphere at –80°C until analysis.

Phospholipid and neutral lipid separation. Because beef muscle tissue contains considerable amounts of neutral lipids, primarily TAG, the neutral lipid and phospholipid fractions were separated by liquid column chromatography, using activated silicic acid as the stationary phase (Clarkson Chemical Company, Inc., Williamsport, PA). Neutral lipids, containing cholesterol, cholesteryl esters, TAG, and DAG, were eluted with 10 vol of chloroform/methanol (58:1 vol/vol) (28). Phospholipids were eluted with 10 vol of methanol. The eluents were stored at –80°C until analysis.

Phospholipid mass determination. Phospholipid mass was determined by assaying for lipid phosphorus content of phospholipid fraction separated by liquid column chromatography as described above (29). After drying down the solvent under a stream of nitrogen, 0.5 mL of water and 0.65 mL of perchloric acid (70%) were added and the tubes heated at 185°C for 1 h. Following digestion, the tubes were cooled to room temperature and 0.5 mL ascorbic acid (10%, wt/vol), 0.5 mL ammonium molybdate (2.5%, wt/vol), and 3.3 mL water were added

and mixed. Color was developed by boiling the mixture for 5 min and, after cooling, the absorbance at 797 nm was measured. Absorbance was converted to mass based on a standard curve.

Cholesterol mass determination. Cholesterol mass of the neutral lipid fraction was determined by an iron-binding assay (30). Briefly, the solvent was removed by evaporation using a stream of N_2 and the lipids were dissolved in 3 mL of ethanol. Then 3 mL of the working solution in concentrated sulfuric acid was added to the ethanol. The working solution was made up by q.s. 8 mL of stock solution containing 2.5% ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in 85% phosphoric acid with concentrated sulfuric acid (H_2SO_4) were added. Color was developed by mixing, and the absorbance at 550 nm was measured. Absorbance was converted to mass based on a standard curve.

Transesterification. Phospholipids and neutral lipids were subjected to base-catalyzed transesterification, converting the acyl chains to FAME. To each fraction, 2 mL of 0.5 M KOH dissolved in anhydrous methanol was added and the samples were incubated at 35°C for 30 min (31). The reaction was quenched with methyl formate, and the FAME were extracted using 2 mL of *n*-hexane. The lower phase was re-extracted two more times with *n*-hexane, and these washes were combined with the original aliquot.

GLC. Individual FAME were separated by GLC by using an SP-2330 column (0.32 mm i.d. × 30 m length) and a Trace GLC (ThermoElectron, Austin, TX) equipped with dual autosamplers and dual FID. FAME were quantified using a standard curve from commercially purchased standards (Nu-Chek-Prep, Elysian, MN); 17:0 was the internal standard (32).

Total RNA isolation and real-time polymerase chain reaction (PCR). Pulverized tissues were shipped on dry ice to The Ohio State University for analysis of gene expression using quantitative real-time reverse transcriptase PCR (RT-PCR). Total RNA from tissue was isolated using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and RNA quality was assessed by agarose gel electrophoresis. Reverse transcription was performed using 1 μg of RNA and M-MLV (Moloney murine leukemia virus RT (Invitrogen); RT condition was 65°C for 5 min, 37°C for 50 min, and 70°C for 15 min. Cyclophilin (CYC), peroxisome proliferator activated receptor gamma (PPARγ), PPARα, and heart-FA binding protein (H-FABP) were quantified by RT-SYBR green real-time PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) and SYBR green as the detection dye. The forward and reverse primers were designed to be located on different exons separated by long introns to prevent the generation of noise signals from contaminated genomic DNA during the PCR reactions. The sequences of the primers used were described as follows: H-FABP-F (5´-CGT GCA GAA GTG GAA TGG ACAA-3´), H-FABP-R (5´-TCT GGT GGC GAG TCC AGG AGT-3´), PPARγ-F (5´-CTG TGA AGT TCA ACG CAC TGGA-3´), PPARγ-R (5´-GGT TCA GCT TGA GCT GCA GCT-3´), PPARα-F (5´-ATC ATG GAA AAG TTC GACT-3´), PPARα-R (5´-TTC TGT AGG TGG AGT TTG AGCA-3´), CYC-F (5´-GTG GTC ATC GGT CTC TTT GG -3´), and CYC-R (5´-CAC CGT AGA TGC TCT TAC CTC-3´). Conditions for real-time PCR were 95°C for 10 min and 40 cycles of 94°C for 15 s, 60°C for 1 min, 72°C for 30 s, and 82°C for 16 s. Real-time PCR was performed in duplicate in 25 μL reactions on the Stratagene MX3000P cycler. The relative level of target gene expression was determined using the comparative $\Delta\Delta C_T$ method for relative quantification with cyclophilin as an endogenous reference.

Statistical analysis Statistical analysis was done using Instat2 from GraphPad (San Diego, CA), using an unpaired, two-way Student's *t*-test, and values were considered significant if $P < 0.05$.

RESULTS

Flaxseed feeding increased muscle n-3 FA. The masses of individual FA in the total phospholipid fraction from Hereford muscle were measured (Table 1). Flaxseed feeding significantly increased the mass of 18:3n-3 (4.0-fold) and increased the elongation products of 18:3n-3, namely, 20:5n-3 (1.4-fold) and 22:5n-3 (1.3-fold). In addition, in the flax-fed group the mass of 18:0 was significantly increased (1.2-fold), as was that of 22:1n-9 (2-fold). There were no observed alterations in 18:2n-6 or 20:4n-6 mass between groups. There was a net increase (1.7-fold) in total n-3 FA in the flax-fed group, which resulted in an elevated n-3/n-6 ratio. Thus, the muscle of Hereford steers fed lignosulfonate-treated flaxseed had significantly elevated n-3 FA mass in the phospholipid fraction.

The FA composition (mol%) of the phospholipid fraction was determined using the data in Table 1, and we also measured the FA composition of the neutral lipid fraction (Table 2). Similar to phospholipids, neutral lipid 18:3n-3 content was significantly elevated (4-fold) by flax feeding. This resulted in a 3-fold increase in the total n-3 FA and an increase in the n-3/n-6 ratio. However, note that PUFA did not account for much of the total FA, rather the saturated and monounsaturated FA accounted for the majority of the FA in the neutral lipid fraction. Flaxseed feeding produced similar changes in neutral and phospholipid 18:3n-3 when expressed as mol%. These data indicate that although the 18:3n-3 content of the neutral lipid fraction was altered by flax feeding, the major source of n-3 FA in the muscle tissue was the phospholipid fraction, owing to the limited amount of long-chain n-3 FA in the neutral lipid fraction.

As in the case of the flax-fed Hereford steers, flaxseed supplementation of the Angus steers significantly increased the mass of 18:3n-3 (4.3-fold) as well as the masses of 20:5n-3 (2.0-fold) and 22:5n-3 (1.2-fold) but the mass of 20:4n-6 was unaltered (Table 3). In contrast to the flaxseed-fed Hereford steers, the mass of 18:0 was not increased by flaxseed intake in the Angus steers, but 18:2n-6 was increased slightly (1.2-fold). There was a net increase (2-fold) in total n-3 FA in the flax-fed Angus group, which resulted in an elevated n-3/n-6 ratio. With respect to FA composition (mol%) of Angus muscle (Table 4), the n-3 FA were elevated 4.3-fold (18:3), 2-fold (20:5), 1.2-fold

TABLE 1
Phospholipid FA Mass in *longissimus dorsi* Muscle of Flaxseed-Fed and Control Hereford Steers[a]

	Control		Flax		Changes	
FAME[b]	Mean	SD	Mean	SD	Decrease	Increase
16:0	35	10	42	6		
16:1	3	1	4	1		
18:0	39	3	47	4*		×1.2
18:1n-9	62	17	60	9		
18:1n-7	9	2	9	3		
18:2n-6	48	11	60	7		
18:3n-6	1	0	1	0		
18:3n-3	3	1	12	1*		×4.0
20:1n-9	1	1	1	0		
20:2n-6	3	1	3	1		
20:3n-6	7	2	8	1		
20:3n-3	0	0	0	0		
20:4n-6	36	3	38	4		
20:5n-3	7	2	10	2*		×1.4
22:1n-9	1	0	2	0*		×2.0
22:4n-6	3	0	3	0		
22:5n-3	15	2	20	2*		×1.3
22:6n-3	2	1	3	1		
Sat	75	13	89	10		
Unsat	200	31	232	21		
MUFA	76	19	76	13		
PUFA	124	14	156	15*		×1.3
n-3	27	5	45	5*		×1.7
n-6	97	12	112	11		
n-3/n-6	0.28	0.05	0.40	0.02*		×1.4
Unsat/Sat	2.68	0.15	2.63	0.09		
PUFA/MUFA	1.73	0.45	2.10	0.39		
MUFA/Sat	1.00	0.11	0.86	0.08*	14%	
PUFA/Sat	1.69	0.22	1.77	0.16		
	$n = 6$		$n = 5$			

[a]All values represent mean ± SD (mg/100 g) with *n* as indicated in the table. The asterisk (*) signifies statistical significance from control samples as determined using an unpaired, two-tailed Student's *t*-test, $P < 0.05$.
[b]Sat, saturated; Unsat, unsaturated; MUFA, monounsaturated FA.

(22:5), and 1.3-fold (22:6) by flaxseed feeding, and 18:0 and 18:2n-6 were also elevated slightly (1.1- and 1.2-fold, respectively). Flaxseed intake decreased the FA 16:0 slightly, and reduced each of the FA 16:1, 18:1n-9, 22:4n-6, and 22:5n-6 about 40%.

Grilling did not decrease n-3 FA content. Because cooking meat may result in a reduction in the n-3 FA, we grilled the steaks from the Angus group on a gas barbecue to a often-used internal temperature of about 64°C. We observed no change in the FA composition of the neutral lipid or phospholipid fraction (Table 5). These data were expressed as mole composition (mol%) because loss of water during the cooking process would alter the FA mass per unit of tissue. We observed a net increase in the total phospholipid and cholesterol mass when normalized to wet weight (Table 6), demonstrating this point. However, the cholesterol-to-phospholipid ratio was unchanged, indicating no adverse changes in these parameters. Note that these data were not corrected for water loss, thus accounting for the changes observed. Thus, the n-3 FA composition of the neutral lipid and phospholipid fractions was not altered by grilling, demonstrating that the enrichment of the n-3 FA is preserved during this cooking process.

Flaxseed feeding increased bovine PPARγ mRNA expressions in muscle tissue. To investigate the effect of feeding flaxseed containing high levels of n-3 FA on H-FABP, PPARγ, and PPARα gene expression in the muscle tissues, quantitative real-time RT-PCR was done (Fig. 1). The PPARγ mRNA expression in the flaxseed-fed Angus steers was increased 2.7-fold compare with control Angus steers, indicating that the increased n-3 FA contents may up-regulate PPARγ in bovine muscle. However, there was no significant difference in the expression levels of H-FABP and PPARα gene between groups.

DISCUSSION

To meet the needs of American beef consumers for a healthier beef product, we examined the ability of two different flaxseed feeding regimens to produce an n-3 FA-enriched beef product. This is not without precedent, and others have demonstrated an enrichment of n-3 FA in cattle fed various forms of n-3 FA (6,8,33–35). In Charolais steers fed for 83 d with flaxseed oil (220 g/d) mixed with soybean and sunflower oils (these at 70 and 8% of the mixture, respectively) that were treated with a formaldehyde-protein treatment to protect PUFA from biohy-

TABLE 2
Phospholipid and Neutral Lipid FA Composition of *longissimus dorsi* Muscle in Flaxseed-Fed and Control Hereford Steers[a]

	Phospholipids					Neutral lipids[b]			
	Control		Flax			Control		Flax	
FAME[c]	Mean	SD	Mean	SD	FAME	Mean	SD	Mean	SD
16:0	14.0	2.2	14.4	0.7	16:0	29.4	1.8	29.3	3.7
16:1	1.4	0.2	1.3	0.4	16:1	5.5	1.0	5.9	0.7
18:0	14.7	1.6	14.6	0.5	18:0	13.9	2.5	13.6	1.5
18:1n-9	22.4	3.1	18.8	1.7*	18:1n-9	47.7	3.7	47.3	4.1
18:1n-7	3.2	0.3	3.0	0.8	18:2n-6	2.5	1.9	2.2	0.9
18:2n-6	17.7	2.3	18.9	1.2	18:3n-3	0.2	0.1	0.8	0.2*
18:3n-6	0.2	0.1	0.2	0.0	20:0	0.1	0.0	0.0	0.0
18:3n-3	1.1	0.2	3.7	0.4*	20:1n-9	0.3	0.1	0.4	0.1
20:1n-9	0.2	0.0	0.2	0.1	20:1n-12	0.1	0.1	0.2	0.0
20:2n-6	1.0	0.1	0.8	0.1*	20:2n-6	0.0	0.0	0.1	0.2
20:3n-6	2.5	0.4	2.3	0.2	20:3n-6	0.0	0.0	0.1	0.1
20:3n-3	0.1	0.1	0.1	0.1	20:4n-6	0.2	0.2	0.1	0.1
20:4n-6	12.5	2.4	11.1	1.2	22:5n-3	0.1	0.1	0.1	0.1
20:5n-3	2.3	0.6	3.1	0.5*	Sat	43.3	2.7	42.9	3.6
22:1n-9	0.3	0.1	0.6	0.1*	Unsat	56.7	2.7	57.1	3.6
22:4n-6	1.0	0.2	0.7	0.1*	MUFA	53.7	4.5	53.7	4.2
22:5n-3	4.8	0.9	5.4	0.5	PUFA	3.0	2.3	3.4	1.5
22:6n-3	0.8	0.3	0.7	0.1	n-3	0.3	0.2	0.9	0.3*
Sat	28.7	1.1	29.1	0.6	n-6	2.7	2.1	2.5	1.3
Unsat	71.3	1.1	70.9	0.6	n-3/n-6	0.13	0.02	0.38	0.08*
MUFA	27.4	3.3	23.8	2.7	Unsat/Sat	1.32	0.15	1.34	0.20
PUFA	43.9	4.1	47.1	3.3	PUFA/MUFA	0.06	0.05	0.06	0.03
n-3	9.1	1.7	13.0	1.3*	MUFA/Sat	1.25	0.19	1.27	0.20
n-6	34.9	3.0	34.1	2.0	PUFA/sat	0.07	0.05	0.08	0.03
n-3/n-6	0.26	0.05	0.38	0.02*		$n = 6$		$n = 5$	
Unsat/Sat	2.50	0.14	2.44	0.08					
PUFA/MUFA	1.65	0.42	2.02	0.38					
MUFA/Sat	0.96	0.10	0.82	0.08*					
PUFA/Sat	1.54	0.20	1.62	0.15					
	$n = 6$		$n = 5$						

[a]All the values represent mean ± SD (mol%) with *n* as indicated in the table. The asterisk (*) signifies statistical significance from control samples as determined using an unpaired, two-tailed Student's *t*-test, $P < 0.05$.
[b]Neutral lipids contain cholesteryl esters, TAG, and DAG.
[c]For abbreviations see Table 1.

drogenation (36), 18:3n-3 mass was 15.6 mg/100 g of tissue (35). The phospholipids values in the present study were comparable with these, as we observed an 18:3n-3 mass of 12 mg/100 g of tissue in the flax-fed Herefords and 13 mg/100 g of tissue in the flax-fed Angus. The mass of 20:5n-3 in the flax-fed Hereford group was similar to that observed by Scollan *et al*. (35), but the flax-fed Angus had a lower level (8.9 mg/100 g). The flax-fed Hereford steers had 39 and 50% higher 22:5n-3 and 22:6n-3 levels, respectively, than observed by Scollan *et al*. (35) in their Charolais steers, but the Angus steers had 10 and 7% lower levels of 22:5n-3 and 22:6n-3 levels than observed in their Charolais steers. Note that 220 g/d of flax oil is equivalent to 630 g/d of flaxseed, which is lower than the amounts used in our study. This suggests that the technique Scollan *et al*. used for protecting flaxseed oil from rumen biohydrogenation was at least partially effective.

In another trial, Scollan *et al*. (34) fed slightly cracked flaxseed to Charolais steers (771 g/d for an average of 120 d), and these animals had total masses of n-3 phospholipid FA (61 mg/100 g muscle) that were 36 and 110% greater than the masses for our Hereford and Angus cattle, respectively. However, neither our attempts to raise n-3 FA levels in beef with a plant source of n-3 FA (α-linolenic acid) nor those of Scollan *et al*. (34,35) or Choi *et al*. (33) have raised the mass of total n-3 FA in bovine muscle as high as those reported for cattle consuming only pasture forage in Australia (155 mg/100 g of rump muscle; 8). The Australian study found that rump and blade cuts of beef had higher total levels of n-3 FA than did strip loin cuts, which is an important finding in light of the fact that most studies related to n-3 enrichment of beef evaluate only loin muscles. A number of factors can cause variation in n-3 FA levels in beef including the particular muscle sampled (7), breed of cattle (33), length of time an n-3 FA source is consumed (6), amount of daily consumption (6), type of dietary source fed (34), and whether the source has some protection from biohydrogenation by rumen microbes (35,37). Several of these factors were different for each study, which may account for the study-to-study variation.

TABLE 3
Phospholipid FA Mass in *longissimus dorsi* Muscle from Flaxseed-Fed and Control Angus Steers[a]

	Control		Flax		Changes	
FAME[b]	Mean	SD	Mean	SD	Decrease	Increase
16:0	32.4	4.0	29.9	5.1		
16:1	2.9	0.5	1.8	0.4*	38%	
18:0	22.5	1.7	25.0	4.9		
18:1n-9	44.7	7.3	26.8	7.9*	40%	
18:1n-7	4.0	0.6	3.9	0.9		
18:2n-6	37.0	5.0	45.2	6.4*		×1.2
18:3n-3	3.0	0.6	13.0	2.6*		×4.3
20:1n-9	0.6	0.4	0.6	0.6		
20:3n-6	4.4	0.6	4.5	0.6		
20:4n-6	26.3	3.0	25.3	3.6		
20:5n-3	2.9	0.6	5.6	0.9*		×2.0
22:4n-6	2.4	0.3	1.4	0.4*	41%	
22:5n-6	0.5	0.2	0.3	0.3		
22:5n-3	7.4	0.8	8.9	1.4*		×1.2
22:6n-3	1.0	0.2	1.2	0.2*		×1.2
Sat	55	5	55	10		
Unsat	137	11	139	22		
MUFA	52	8	33	9*	36%	
PUFA	85	8	106	15*		×1.2
n-3	14	2	29	5*		×2.0
n-6	71	8	77	11		
n-3/n-6	0.20	0.04	0.37	0.03*		×1.9
Unsat/Sat	2.50	0.07	2.54	0.05		
PUFA/MUFA	1.66	0.31	3.29	0.53*		×2.0
MUFA/Sat	0.95	0.09	0.60	0.08*	37%	
PUFA/Sat	1.55	0.14	1.94	0.11*		×1.2
	$n = 10$		$n = 10$			

[a]All values represent mean ± SD (mg/100 g tissue) with *n* as indicated in the table. The asterisk (*) signifies statistical significance from control samples as determined using an unpaired, two-tailed Student's *t*-test, $P < 0.05$.
[b]For abbreviations see Table 1.

Although flaxseed is a good source of plant-based n-3 FA, others have fed cattle fish meal-based rations. In one of these studies, feeding fish meal to cattle for 112 d elevated 20:5n-3 and 22:6n-3 levels in bovine *longissimus* muscle considerably higher than seen by feeding flaxseed meal or oil alone (6). Others have observed somewhat higher 20:5n-3 and 22:6n-3 levels in bovine muscle when a combination of flaxseed and fish oil was fed (33). These results indicate that if higher 20:5n-3 and 22:6n-3 levels are desired in bovine muscle, sources of 20:5n-3 and 22:6n-3 may have to be fed rather than relying solely on synthesis *de novo* of 20:5n-3 and 22:6n-3 from 18:3n-3. Dietary 18:3n-3 is probably the direct precursor for the synthesis *de novo* of 20:5n-3, 22:5n-3, and 22:6n-3 (34,35), and our previous results in rats with 18:3n-3 feeding indicates tissue selectivity with regard to elongation and accretion of 18:3n-3-derived FA such as 20:5n-3, 22:5n-3, and 22:6n-3 (38). However, it is conceivable that greater elevations in muscle 20:5n-3, 22:5n-3, and 22:6n-3 content may occur either by increasing the conversion of 18:3n-3, by increasing the duration of 18:3n-3 feeding, or by using cattle selected for finishing on forage, such as seen in Australian beef (8).

In hogs, feeding flaxseed produces much different results compared with those for cattle. Feeding hogs crushed flaxseed (6% of the ration) for 60 d prior to slaughter increased the mass of 18:3n-3 and 20:5n-3 in phospholipids of pork *longissimus* muscle 3.8- and 2.8-fold, respectively (14). A higher ground flaxseed content (15% of ration) fed to pigs for 42 d had an even greater increase in phospholipid 18:3n-3 and 20:5n-3 mass of pork *longissimus* muscle: 11.7- and 3.2-fold, respectively (13). Clearly, in hogs it is much easier to enrich the muscle in 18:3n-3 and 20:5n-3 by including flaxseed in the rations than it is in cattle. Microbial fermentation of feedstuffs before the true stomach of cattle and the consequent low level of n-3 FA available to their muscle cells compared with no microbial fermentation of digesta before the stomach of pigs likely explains differential enrichment potential for swine vs. cattle. However, similar to the results in cattle, hogs have limited conversion of 18:3n-3 to longer-chain n-3 FA in muscle. Certainly in cattle, the major longer-chain n-3 FA is 22:5n-3, suggesting no biological need for the muscle to elongate 22:5n-3 to 22:6n-3, consistent with our observations here and in rats (38).

Cooking meat may decrease the PUFA content due to oxidation or loss in drippings. When comparing cooked to uncooked meat, one must take into account the water loss observed during cooking. Our approach was to express our data in mol%, thereby negating the need to account for water loss, as this parameter is independent of the mass of the starting material. Our results with grilled steaks are consistent with others

TABLE 4
Phospholipid FA Composition in *longissimus dorsi* Muscle from Flaxseed-Fed and Control Angus Steers[a]

	Control		Flax		Changes	
FAME[b]	Mean	SD	Mean	SD	Decrease	Increase
16:0	18.4	0.9	17.1	0.7*	7%	
16:1	1.7	0.2	1.0	0.1*	41%	
18:0	11.7	0.5	12.9	0.7*		×1.1
18:1n-9	23.3	2.9	13.8	2.1*	40%	
18:2n-6	19.4	2.2	23.5	1.8*		×1.2
18:3n-3	1.6	0.2	6.8	0.4*		×4.3
20:1n-9	0.3	0.2	0.3	0.2		
20:2n-6	0.2	0.0	0.2	0.0		
20:3n-6	2.2	0.3	2.1	0.2		
20:4n-6	12.8	1.2	12.1	1.0		
20:5n-3	1.4	0.3	2.8	0.4*		×2.0
22:4n-6	1.1	0.1	0.6	0.1*	40%	
22:5n-6	0.2	0.1	0.1	0.1*	42%	
22:5n-3	3.3	0.4	4.1	0.4*		×1.2
22:6n-3	0.4	0.1	0.6	0.1*		×1.3
Sat	30.1	0.6	30.0	0.8		
unsatU	69.9	0.6	70.0	0.8		
n-3	6.7	0.8	14.2	0.9*		×2.0
n-6	35.8	3.5	38.7	2.5*		×1.1
n-3/n-6	0.19	0.04	0.37	0.04*		×1.9
Unsat/Sat	2.32	0.06	2.34	0.09		
PUFA/MUFA	1.59	0.30	3.15	0.49*		×2.0
MUFA/Sat	0.91	0.08	0.57	0.07*		
PUFA/Sat	1.42	0.13	1.77	0.12*		×1.2
	$n = 10$		$n = 10$			

[a]All values represent mean ± SD (mol%) with *n* as indicated in the table. The asterisk (*) signifies statistical significance from control samples as determined using an unpaired, two-tailed Student's *t*-test, $P < 0.05$.
[b]For abbreviations see Table 1.

demonstrating that cooking of the *longissimus* muscle to a final internal temperature of 65°C does not alter the FA composition (25). However, another study showed a small increase in 18:3n-3 content in beef patties cooked to an internal temperature of 77°C, although this study did not examine longer-chain n-3 FA (24). In contrast, another study with *longissimus* muscle demonstrated that cooking to an internal temperature of 70°C resulted in substantial loss of 18:3n-3 and a loss of PUFA in general (23). This report is similar to a study with grilled rump steak cooked to "rare," "medium," or "well-done," where there were no changes in 18:3n-3 content but a decrease in 20:5n-3 and 22:6n-3 contents when the meat was cooked beyond "rare" (22). However, in the study of Sinclair et al. (22), the content of 22:5n-3 was reduced if the steaks were cooked at all, whereas in our study, we demonstrate no change in phospholipid FA composition. In addition, when the values are expressed as mass, there is a clear increase in n-3 FA content (data not shown) similar to that observed in total phospholipid mass (Table 6).

Dietary FA are well known regulators of gene expression (39–41), acting through a number of different transcriptional activators including the PPAR family. Both n-3 and n-6 FA are regulators of gene transcription (41), with 20:5n-3 and 22:6n-3 inducing gene expression through a PPARγ-mediated process (42). PPARγ activation is associated with reduced late-stage progression of type-2 diabetes (43,44), a well-known problem associated with feedlot cattle production. Because the PPAR family regulates expression of FA transport proteins (45) and because n-3 FA stimulate this PPAR-mediated process (42), we examined the impact of n-3-enriched diets on PPAR expression in muscle. In addition, we examined the impact of this dietary regimen on H-FABP (FABP3) expression, a protein associated with FA uptake in muscle (46). We found that PPARγ mRNA level was elevated 2.7-fold in the muscle from the flaxseed-fed cattle, whereas PPARα and FABP3 mRNA levels were unchanged. These results suggest that this feeding regimen may have potential downstream protection of cattle from complications associated with type-2 diabetes. In addition, it suggests a potential change in the PPARγ regulated gene-expression, known to have an impact on genes associated with lipid metabolism (40,41) and inflammation (42).

In summary, n-3 levels in the *longissimus* muscle from Hereford and Angus cattle were clearly enriched by feeding flaxseed. The n-3 FA mass was maintained during cooking, demonstrating that the enrichment was preserved during the cooking process. Overall, these results represent an important step in providing Americans an n-3 FA-enriched beef product. In addition, our n-3 feeding regimen increased expression of PPARg in muscle, suggesting long-term feeding may change gene expression for proteins involved in lipid metabolism.

TABLE 5
Phospholipid and Neutral Lipid FA Composition of Raw and Grilled Steaks (*longissimus dorsi* muscle) Obtained from Flaxseed-Fed Angus Steers[a]

	Phospholipids					Neutral lipids[b]			
	Uncooked		Cooked[c]			Uncooked		Cooked[c]	
FAME[d]	Mean	SD	Mean	SD	FAME	Mean	SD	Mean	SD
16:0	17.7	0.8	17.4	0.9	16:0	29.3	2.1	28.5	1.8
16:1	1.0	0.2	1.1	0.2	16:1	3.7	0.8	3.2	0.6
18:0	13.2	0.5	12.9	0.5	18:0	17.1	2.8	18.7	2.4
18:1n-9	15.2	2.1	15.3	2.0	18:1n-9	46.6	1.5	46.1	1.1
18:1n-7	1.9	0.1	1.9	0.3	18:2n-6	1.5	0.3	1.7	0.3
18:2n-6	22.6	1.7	23.0	1.6	18:3n-3	0.7	0.2	0.7	0.1
18:3n-6	0.3	0.0	0.3	0.0	20:0	0.1	0.0	0.0	0.0
18:3n-3	5.5	0.5	5.5	0.4	20:1n-9	0.4	0.1	0.4	0.1
20:0	0.1	0.0	0.1	0.0	20:1n-12	0.2	0.1	0.2	0.1
20:1n-9	0.2	0.1	0.2	0.0	20:2n-6	0.1	0.1	0.1	0.0
20:1n-12	0.1	0.0	0.1	0.1	20:4n-6	0.1	0.0	0.1	0.0
20:2n-6	0.3	0.0	0.3	0.0	22:5n-3	0.1	0.0	0.1	0.0
20:3n-6	2.2	0.3	2.3	0.2	22:6n-3	0.1	0.1	0.1	0.0
20:4n-6	11.5	1.0	11.3	0.9	Sat	46.5	1.6	45.5	1.2
20:5n-3	2.8	0.3	2.8	0.3	Unsat	53.5	1.6	54.5	1.2
22:1n-9	0.1	0.1	0.2	0.0	MUFA	50.8	1.9	51.7	1.5
22:3n-3	0.6	0.1	0.7	0.1	PUFA	2.7	0.5	2.9	0.4
22:5n-3	4.0	0.2	4.1	0.3	n-3	0.9	0.2	0.8	0.1
22:6n-3	0.6	0.1	0.6	0.1	n-6	1.8	0.3	2.1	0.3
Sat	31.0	1.0	30.4	0.8	n-3/n-6	0.52	0.03	0.39	0.03*
Unsat	69.0	1.0	69.6	0.8	Unsat/Sat	1.15	0.07	1.20	0.06
MUFA	18.7	2.4	18.7	2.1	PUFA/MUFA	0.05	0.01	0.06	0.01
PUFA	50.4	3.3	50.8	2.6	MUFA/Sat	1.10	0.08	1.14	0.06
n-3	13.4	0.8	13.7	0.8	PUFA/Sat	0.06	0.01	0.06	0.01
n-6	36.9	2.8	37.2	2.4					
n-3/n-6	0.36	0.02	0.37	0.03					
Unsat/Sat	2.23	0.11	2.29	0.08					
PUFA/MUFA	2.76	0.51	2.76	0.46					
MUFA/Sat	0.60	0.06	0.61	0.06					
PUFA/Sat	1.63	0.16	1.67	0.12					

[a]All values represent means ± SD (mol%), $n = 10$. The asterisk (*) signifies statistical significance from uncooked samples as determined using an unpaired, two-tailed Student's *t*-test, $P < 0.05$.
[b]Neutral lipids contain cholesteryl esters, TAG, and DAG.
[c]Ribeye steaks were cooked to an internal temperature of 64.1 ±± 0.4°C.
[d]For abbreviations see Table 1.

TABLE 6
Phospholipid and Cholesterol Content in Raw and Grilled Steaks (*longissimus dorsi* muscle) Obtained from Flaxseed-Fed Angus Steers[a]

	Uncooked		Cooked[b]	
	Mean	SD	Mean	SD
Phospholipid[c]	6358	569	9573	1187*
Cholesterol[c]	2397	453	3177	356*
Cholesterol/phospholipid[d]	0.38	0.08	0.34	0.06

[a]All values represent means ± SD (nmol/g wet weight), $n = 10$. The asterisk (*) signifies statistical significance from uncooked samples as determined using an unpaired, two-tailed Student's *t*-test, $P < 0.05$.
[b]Ribeye steaks were cooked to an internal temperature of 64.1 ± 0.4°C.
[c]Not corrected for water loss.
[d]The cholesterol/phospholipid ratio represents total nmol of cholesterol divided by the total nmol of phospholipid.

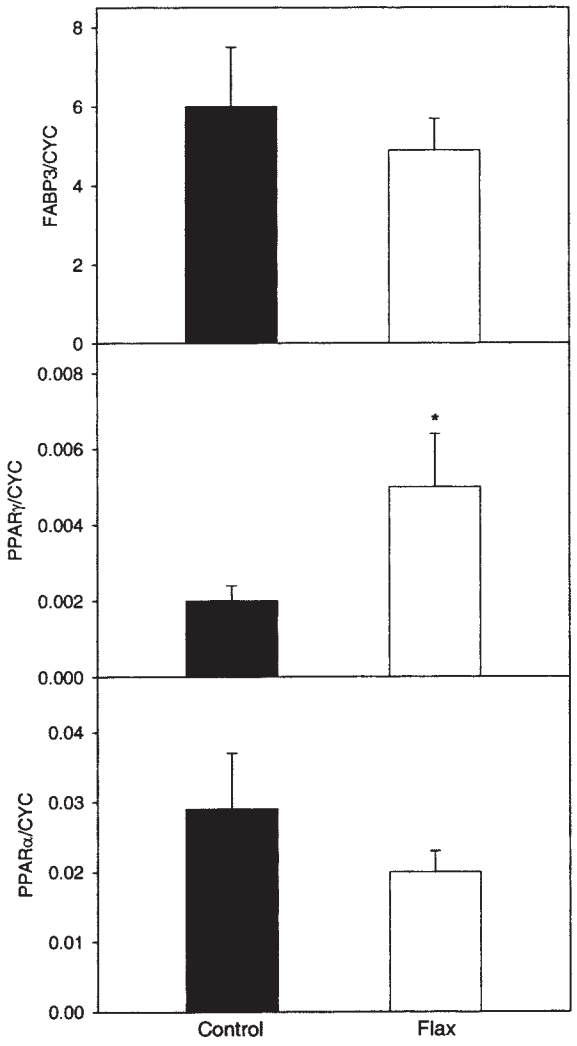

FIG. 1. The effect of flaxseed-feeding on the heart-FA binding protein (FAPB3), peroxisome proliferator activated receptor γ (PPARγ), and PPARα mRNA levels in *longissimus dorsi* muscle of Angus steers. Values represent means and SD, $n = 10$. The asterisk (*) indicates significance from control, $P < 0.05$. CYC, cyclophilin.

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Diacylglycerol Acyltransferase: A Key Mediator of Plant Triacylglycerol Synthesis

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ABSTRACT: Many plants deposit TAG in seeds and fruits as the major form of storage lipid. TAG production is of tremendous socioeconomic value in food, nutraceutical, and industrial applications, and thus numerous conventional and molecular genetic strategies have been explored in attempts to increase TAG content and modify the FA composition of plant seed oils. Much research has focused on the acyl-CoA–dependent reaction catalyzed by diacylglycerol acyltransferase (DGAT), which is an integral endoplasmic reticulum protein and has also been shown to be present in oil bodies and plastids. DGAT enzymes exhibit diverse biochemical properties among different plant species, many of which are summarized here. In addition to catalyzing a critical step in TAG biosynthesis, there is evidence that DGAT has roles in lipid metabolism associated with germination and leaf senescence. TAG can also be formed in plants via two different acyl-CoA–independent pathways, catalyzed by phospholipid:diacylglycerol acyltransferase and diacylglycerol transacylase. The current understanding of the terminal step in TAG formation in plants and the development of molecular genetic approaches aimed at altering TAG yield and FA composition of TAG are discussed.

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In a number of plants, TAG are the major storage lipids that constitute a highly reduced form of carbon and serve as an important energy reserve in seeds for subsequent germination and seedling development. These storage lipids are of great nutritional and nutraceutical value, and thus a common source of edible oils for human consumption. They are also becoming increasingly important raw materials in the petrochemical industry for the production of paints, detergents, lubricants, biofuels, and nylon precursors, as it is an undisputable fact that non-renewable crude oil reserves are rapidly being exhausted. Because of our heavy reliance on plant oils in the food and petrochemical industries, boosting the seed oil content and generating desirable FA in oilseed crops are of tremendous socioeconomic value. The implementation of molecular genetic strategies to achieve these goals necessitates a comprehensive understanding of TAG synthesis in plants.

Formation of storage lipids in plants involves *de novo* FA synthesis in the stroma of plastids and subsequent incorporation of the FA into glycerol backbone leading to TAG in the endoplasmic reticulum (ER). The present review sets out to evaluate the terminal step in TAG formation in plants. This committed step primarily involves the catalytic action of diacylglycerol acyltransferase (DGAT; EC 2.3.1.20), which acylates DAG to form TAG in an acyl-CoA–dependent manner. The subcellular localization, biochemical properties, molecular biology, and physiological roles of this enzyme are reviewed in the present article. Recently, acyl-CoA–independent reactions leading to TAG formation have also been described. Current understanding of the two involved enzymes, phospholipid:diacylglycerol acyltransferase (PDAT; EC 2.3.1.158) and diacylglycerol transacylase (DGTA), is also evaluated. This review begins with a brief description of storage lipid synthesis in plants.

STORAGE LIPID SYNTHESIS IN DEVELOPING SEEDS

A generalized scheme of TAG synthetic pathways in developing seeds is depicted in Figure 1. FA synthesis in plants has been extensively reviewed elsewhere (1,2). In plastids, FA are synthesized from acetyl-CoA in a three-step process: (a) irreversible carboxylation of acetyl-CoA by the action of acetyl-CoA carboxylase (EC 6.4.1.2) to form malonyl-CoA; (b) repeated condensation of malonyl-CoA with a growing acyl carrier protein (ACP)-bound acyl chain by the action of FA synthase complex, consecutively adding two carbon units to form 16:0-ACP; and (c) elongation and desaturation of 16:0-ACP to form 18:0-ACP and 18:1-ACP, respectively. The *de novo* synthesized FA enter the cytosolic pool in an esterified form known as acyl-CoA, which are synthesized by an ATP-dependent esterification of FA and CoA through the action of acyl-CoA synthetase (EC 6.2.1.3). In erucic acid–rich *Brassica* species, the acyl-CoA pool is further enriched with 22:1-CoA by the action of membrane-bound FA elongation (FAE) complex on the ER.

In the ER, the sequential incorporation of FA onto the glycerol backbone is commonly known as the Kennedy pathway (3). The pathway starts with the acyl-CoA–dependent acylation of *sn*-glycerol-3-phosphate to form lysophosphatidic acid through the action of *sn*-glycerol-3-phosphate acyltransferase (EC 2.3.1.15). The second acyl-CoA–dependent acylation is

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Abbreviations: ACP, acyl carrier protein; ASP, acylation stimulating protein; ATP, adenosine triphosphate; CDP, cytidine diphosphate; CPT, cytidine diphosphate-choline:1,2-diacylglycerol cholinephosphotransferase; DGAT, DAG acyltransferase; DGTA, DAG transacylase; ER, endoplasmic reticulum; EST, expressed sequence tag; FAD, FA desaturation; GUS, β-glucuronidase; MD, microspore-derived; MEGA-8, octanoyl-*N*-methylglucamide; PDAT, phospholipid:diacylglycerol acyltransferase.

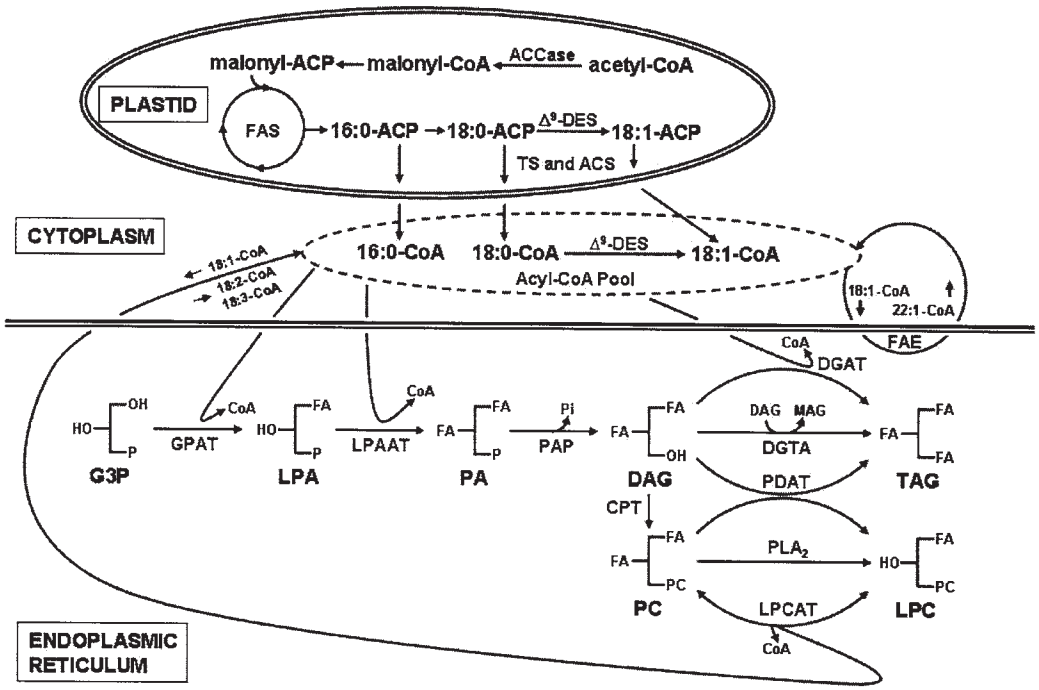

FIG. 1. Generalized scheme of FA and TAG synthesis in developing seeds of oleaginous plants. FA synthesis occurs in plastids. The *de novo* synthesized FA enter the cytosolic acyl-CoA pool, which fuels the assembly of TAG from *sn*-glycerol-3-phosphate (G3P) in the ER. PUFA are enriched in the acyl-CoA by the action of desaturase at the level of PC and lysophosphatidylcholine acyltransferase (LPCAT)-mediated acyl exchange. In *Brassica* species, erucic acid is produced by two-step elongation of oleoyl-CoA. Δ^9-DES, Δ^9-desaturase; ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; ACS, acyl CoA synthetase; CPT, CDP-choline: 1,2-diacylglycerol cholinephosphotransferase; DGAT, diacylglycerol acyltransferase; DGTA, diacylglycerol transacylase; FAE, FA elongase; FAS, fatty acid synthase; GPAT, *sn*-glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; PAP, phosphatidate phosphatase; PDAT, phospholipid:diacylglycerol acyltransferase; PLA_2, phospholipase A_2; TS, acyl-ACP thioesterase.

catalyzed by lysophosphatidic acid acyltransferase (EC 2.3.1.51), leading to the formation of PA, which is dephosphorylated through the action of phosphatidate phosphatase (EC 3.1.3.4) to form *sn*-1,2-DAG. The third acyl-CoA–dependent acylation catalyzed by DGAT leads to the production of TAG. DAG is situated at the branch point of the pathway between TAG and membrane phospholipid formation. Cytidine diphosphate (CDP)-choline:1,2-DAG cholinephosphotransferase (CPT; EC 2.7.8.2) catalyzes the transfer of a phosphocholine from CDP-choline into DAG and leads to the formation of PC and cytidine monophosphate.

PUFA are enriched in the acyl chains of DAG by the FA desaturation (FAD) pathways catalyzed by FAD2 and FAD3 desaturases that utilize PC as substrate (4) and the reverse reaction of CPT that channels PC back to DAG. The acyl moiety at the *sn*-2 position of PC may undergo acyl exchange with the acyl-CoA pool by the reversible reaction catalyzed by lysophosphatidylcholine acyltransferase (EC 2.3.1.23). Lysophosphatidylcholine can also be formed through hydrolysis of PC catalyzed by phospholipase A_2 (EC 3.1.1.4). Recent studies have indicated that TAG could also be formed in acyl-CoA–independent reactions using DAG and PC as the acyl donors (5,6). PDAT catalyzes the acyl transfer from PC to DAG leading to the formation of lysophosphatidylcholine and TAG, whereas DGTA catalyzes the transfer of an acyl moiety between two DAG molecules to form TAG and monoacylglycerol (MAG).

SUBCELLULAR LOCALIZATION OF DGAT

The subcellular localization of DGAT has been a subject of controversy for decades. Numerous earlier studies have demonstrated the presence of DGAT activity in oil bodies (7), which have been regarded as the principal site of TAG synthesis (8–15). Subsequently, other researchers, however, argued that DGAT is associated with the ER (16–19). By retaining the attachment of polysomes to the membrane fractions in the presence of Mg^{2+} during isolation, Cao and Huang (20) were able to localize the maize DGAT to the rough ER.

Although there is evidence for DGAT activity in both oil bodies and ER, the debate over how DGAT ends up in oil bodies can be related to investigations of the ontogeny of the oil bodies. The widely accepted theory is that oil bodies are continuous with and generated from the DGAT-harboring ER (21,22), leading to the persistence of DGAT in the membranes of oil bodies after budding (23). This theory has been supported

by electron microscopy observations in developing oilseeds that indicated that sphereosomes budded off from the terminal strands of ER and developed into oil bodies (20), and that oil bodies were frequently in proximity to the ER (24), implicating their *in vivo* association. Thus, the *de novo* biosynthesis of TAG in purified oil body fractions could be due to their contamination with membrane fractions (25). For instance, DGAT from oil bodies and membrane fractions have been found to share similar enzymic properties (11). The purified oil bodies from germinating soybean cotyledons also exhibited activities for ER markers, suggesting *in vivo* association of the two organelles (24).

On the other hand, some researchers have hypothesized that oil bodies are independent of the ER, exuded into the cytoplasm in the form of naked droplets and subsequently coated with oleosins and phospholipids (14,18,26–28). In this context, earlier studies with acyltransferases in crambe (*Crambe abyssinica*) seeds indicated the presence of enzymic activities in oil body, nuclear/plastid, and mitochondrial fractions with apparent absence of DGAT activity in microsomal fractions (29). The most critical cue that favors the second theory is the considerably higher DGAT activity in the oil body fractions of certain plant materials than in the microsomal fractions in terms of specific (9,11,13,15) and total (11) activity. Concomitantly, a recent investigation of DGAT in oil bodies purified from freshly excised maize developing embryos indicated that the enzyme activity in this organelle was too high to be completely ascribed to microsomal contamination and may have been underestimated in the absence of endogenous DAG for enzyme assays (30).

Nonetheless, Kamisaka and Nakahara (11) argued that the floating membrane-bound DGAT might be drawn into the lipid-rich oil body fraction due to a high enzyme-lipid affinity, leading to the high occurrence of artificial DGAT activity in this fraction. Lacey and Hills (17) clearly showed the heterogeneity of ER in developing seeds of *Brassica napus* L. with respect to lipid synthesis, from which they proposed another explanation that DGAT might concentrate in certain TAG-secreting ER regions separated from the bulk ER by their lower equilibrium density.

Despite the controversy regarding oil body ontogeny, the ER is believed to be the principal site of TAG synthesis in developing oilseeds (25). In fact, recent studies of DGAT have extensively utilized the microsomal fractions from developing seeds of many plants as well as the microspore-derived (MD) culture of *B. napus* (31). Recently, Shockey *et al.* (32) further localized two unrelated tung DGAT activities (type 1 and type 2) to non-overlapping, distinct subdomains of ER using immunofluorescence staining. The subcellular localization of both enzymes to the ER is attributable to the presence of ER retrieval motifs at C-termini, whereas the targeting of both enzymes to their respective ER subdomains requires additional protein sequences (32).

In addition to the subcellular localization of DGAT in oil bodies and the ER, this protein has also been found in the chloroplasts of spinach leaves (33) and soybean cell suspension cultures (34). Plastidial DGAT may account for the presence of a trace amount of TAG in chloroplast envelopes (33,35,36) and plastoglobuli (oil bodies in stroma) (33). The relatively low DGAT activity in the microsomal fractions from photoautotrophic cells (33,34) is in agreement with the suggestion that lipid assembly in chloroplasts is self-sufficient (37), although it is plausible that certain lipids are imported from the ER to the thylakoids and envelopes (38,39). Interestingly, TAG assembly from palmitic acid, exogenously applied to the upper surface of spinach leaves, was evident, confirming the activity of plastidial DGAT *in vivo* (40). Recently, immunochemical techniques were used to demonstrate that DGAT is localized to intact chloroplasts, purified thylakoids, and envelope membranes from *Arabidopsis* leaves, but it is absent from the stroma (41).

OPTIMIZATION OF *IN VITRO* DGAT ASSAYS

The role of exogenous DAG as an acyl acceptor varies with different assay systems. It has been reported that DGAT activity in maize microsomes was reduced by one-third in the absence of exogenous DAG (20). Concomitantly, supplementation of exogenous DAG enhanced DGAT activity in the particulate fractions from maturing safflower seeds (42,43) and microsomal and oil body fractions from palm mesocarp (15). Other researchers have reported, however, that the effect of exogenous DAG on *in vitro* DGAT activity was not substantial (17,30,35,44–48), presumably due to the adequate amount of DAG endogenously present in the enzyme preparations or produced during the course of incubation (20,23,48). The degree of enzyme dependency on exogenous DAG takes into account the endogenous DAG content, which varies with the plant species and the stage of seed development (43), as well as the accessibility of the exogenous DAG to the enzyme (49). The degree of dependence on endogenous DAG is probably also affected by the molecular species of DAG present. By dispersion of the exogenous DAG in a suitable detergent (e.g., Triton X-100), DGAT activity was found to be enhanced in association with an increasing amount of exogenous DAG (49). Valencia-Turcotte and Rodríguez-Sotres (30) also observed that the octanoyl-*N*-methylglucamide (MEGA-8)-solubilized DGAT was notably more active after the addition of emulsified DAG. Commonly used DAG substrates and assay concentrations of substrate are listed in Table 1.

TABLE 1
Different Acyl Accepters Used in *in vitro* DGAT Assays

Acyl accepter	Concentration (mM)	References
Diolein	0.33	(46–48,81,108,109)
	0.1	(75)
	0.2	(53)
	1	(49,130)
	1.6	(80,114)
	2	(20,71)
Dipalmitin	0.05	(9)
	1.5	(15)
1-Palmitoyl-2-oleoyl glycerol	5	(72)

TABLE 2
Different Acyl Donors Used in *in vitro* DGAT Assays

Acyl donor	Concentration (μM)	References
[1-^{14}C] Oleoyl-CoA	1.6	(42)
	3.4	(49,130)
	14	(48,81)
	15	(46,47,67,108,109)
	18	(80,114,131)
	20	(20,45)
	25	(35)
	30	(30)
	40	(53)
[9,10-^{3}H] Oleoyl-CoA	65	(65)
[1-^{14}C] Palmitoyl-CoA	0.39	(15)
	25	(35)
	200	(9)
[1-^{14}C] Stearoyl-CoA	18	(80)
	25	(35)
[1-^{14}C] Erucoyl-CoA	14	(81)

TABLE 3
Detergents Used in *in vitro* DGAT Assays

Detergent	Concentration (%, wt/vol)	References
Tween-20	0.02	(46,48,81)
	0.04	(20,30,71)
	0.05[a]	(9)
	0.1	(72,108,109)
	0.2	(47)
Triton X-100	0.1	(11,49)
	2	(112)
Zwitteronic detergent 3-08	0.02	(80)

[a]Added in a mixture.

Radiolabeled acyl-CoAs were routinely supplied as acyl donors in most *in vitro* assays of DGAT activity (Table 2). Hobbs and Hills (45) demonstrated that omission of acyl-CoA led to a 10-fold reduction in DGAT activity. Radiolabeled TAG product can be quantified following separation by TLC or using radio-HPLC (48,50). Radiolabeled DAG can be used only if the label is located on the glycerol moiety because FA-labeled DAG is subject to possible interference by acyl exchange reactions (31,51). Assays conducted using radiolabeled acyl-CoA, however, are not subject to interference by acyl-CoA–independent processes including the reactions catalyzed by DGTA and PDAT (52).

In an assay system, the reactants and enzymes can be dispersed by sonication (30,53) or detergents such as Tween-20, Triton X-100, and Zwitteronic detergent 3-08 (Table 3). Selecting a suitable detergent in an assay system takes into consideration the compromise between its solubilizing ability as well as its denaturing effect on the enzyme, both of which vary with different DGAT samples. For instance, Triton X-100 was routinely used in some studies (Table 3), and it was found to be inhibitory to the activity of other DGAT preparations (35,42,43), although Cao and Huang (20) later demonstrated that the inhibitory effect of Triton X-100 could be minimized by pretreatment of the enzyme preparation with the same detergent. Other detergents including sodium deoxycholate, sodium dodecyl sulfate, and sucrose monolaurate have been shown to abolish enzyme activity (20,42,49).

SUBSTRATE SPECIFICITY AND SELECTIVITY OF DGAT

It is possible that some DGAT can utilize acyl acceptors other than DAG such as sterols. A DGAT mutant in *Arabidopsis* has not been shown, however, to exhibit any abnormality in steryl ester proportion compared to the wildtype (54). Similarly, Bouvier-Navé *et al.* (23) have shown that overexpression of a DGAT-encoding cDNA from *Arabidopsis* (*AtDGAT1*) did not complement the knocked-out acyl-CoA:cholesterol acyltransferases (ACAT; EC 2.3.1.26) in a yeast double-mutant, which exhibited similar sterol profiles and ACAT activity, despite the relatively high homology between the encoding sequences of the two enzymes (55). It is interesting to note, however, that fungal (*Mortierella ramanniana*) (56) and mouse (57) DGAT can acylate MAG, whereas the latter also exhibited activity with wax monoesters, diesters, and retinyl esters (57).

Ichihara and Noda (43) reported that DGAT activity in the particulate fraction from maturing safflower seed utilized *sn*-1,2-DAG and *sn*-2,3-DAG, but was apparently inactive with *sn*-1,3-DAG. The acyl composition of DAG affects DGAT activity differently depending on the source of the enzyme. A relatively broad preference for molecular species of DAG has been observed in some studies (43,58). DAG species with very long chain FA were preferred in certain nasturtium (59) and rape varieties (60,61). DGAT-catalyzed TAG synthesis in spinach leaves was considerable with diolein, but hardly detectable with dipalmitin (35). Water-soluble DAG including *rac*-1,2-diacetylglycerol and *rac*-1,2-dibutyrylglycerol are poor substrates of DGAT from safflower seeds (43). Vogel and Browse (58) demonstrated that microsomal DGAT from *Cuphea* and castor bean exhibited high activity toward DAG containing unusual FA (e.g., lauric and ricinoleic acids) at both *sn*-1 and *sn*-2 positions. The investigation proposed that rapid conversion of unusual DAG species to TAG might be a mechanism of plants to restrict the entry of unusual FA into PC and other membrane phospholipids.

The abundance of medium-chain and hydroxyl FA in *Cuphea* seeds (62,63) and castor beans (63,64) has been attributed to the high selectivity of their respective DGAT for DAG species containing these FA. Although it has been suggested that the substrate specificity of DGAT might not be an important factor for determining the acyl composition of TAG (56), a precise conclusion on the preference of specific DGAT for specific DAG species certainly necessitates a survey of more DGAT from various sources, which has been hindered by the difficulties associated with removing endogenous DAG in the enzyme preparations (35,46). Sukumar and Sastry (65) removed the endogenous DAG in a microsomal preparation from developing groundnut seeds by high-salt (4 M KCl) extraction, dialysis, and precipitation.

It is generally believed that DGAT exhibits absolute specificity to acyl-CoA as the acyl donor, despite the limited evidence obtained from a fungal (*Mortierella ramanniana*) DGAT, which did not acylate with PC, DAG, or oleic acid (56). Inter-

TABLE 4
Acyl-CoA Specificity of Microsomal DGAT from Various Plant Sources

	Acyl-CoA specificity									
Plant species	12:0	16:0	18:0	18:1	18:2	18:3	22:1	20:5	22:6	References
Rape	2			1			3			(71)
				2			1			(48,73)
		3		2			1			(9)
		1	3	2						(46)
Maize		2		3	1					(20)
	2			1			N			(71)
Soybean		2		3	1					(20)
Peanut		1		3	2					(20)
Castor bean		1		2	3					(20)
Cuphea	1			2			N			(71)
Sunflower		1	N	1						(16)
Oil palm		2	3	1						(15)
Flax				3	2	1		4	5	(47)
Canola	1			2			3			(71)
Spinach[a]		1	2	3						(35)

For each sample, DGAT specificity is ranked in descending order of activity: 1, highest; 5, lowest; N: negligible.
[a]DGAT was obtained from leaves

estingly, Shine *et al.* (66) claimed that an enzyme preparation from avocado mesocarp microsomes could catalyze the incorporation of an acyl moiety from ACP thioesters into the *sn*-3 position of TAG. In contrast to the rather similar acyl composition in leaf tissues, tremendous variability in acyl composition and occurrence of unusual FA are mostly confined to oilseeds (8). Accordingly, the biochemical characterization of DGAT from developing seeds has mostly, if not exclusively, dealt with the acyl-CoA preference of the enzyme. Substrate preference of DGAT is studied with respect to specificity and selectivity determinations. The former is studied using a single acyl-CoA species at a time, whereas a mixture of acyl-CoA species is utilized in the measurement of the latter, which is generally regarded as a more realistic reflection of the *in vivo* situation (31). An equimolar mixture of different acyl-CoA species is commonly used in a selectivity study, which in certain cases has also been carried out with different ratios of thioesters (67). For a better assessment of the substrate preference of DGAT (68), complete assembly of TAG from exogenously applied *sn*-glycerol-3-phosphate has also been studied (60,68,69).

In some plant species, different acyl-CoA species can hardly be differentiated by DGAT (43,67), whereas other plant DGAT preferentially utilize certain acyl-CoA species (Tables 4 and 5). The acyl-CoA selectivity of DGAT depends on several factors, including acyl composition of the DAG pool (32,70), acyl-CoA concentration (48), and temperature (2,8,19,71,72). It has been reported that the incorporation of saturated FA into glycerolipids was preferred at higher temperature in a study over a range of 15–40°C using cacao microsomes (72). The temperature effect on acyl-CoA selectivity of DGAT has been attributed to the differential changes in substrate-enzyme affinity and solubility of the substrates (71).

In general, plant DGAT exhibit a broad acyl-CoA preference as revealed by the specificity (Table 4) and selectivity studies (Table 5). As a result, it is believed that the acyl composition of TAG in oilseeds is predominantly controlled by other factors such as availability of specific DAG and acyl-CoA pools to the enzymes (56). In some cases, however, the substrate preference of DGAT does play a decisive role in the specific channelling of certain FA into TAG molecules (16), exemplified by the selectivity of cocoa DGAT for stearoyl-CoA (68) and the strong preference of DGAT from certain rape varieties for erucoyl-CoA (9,48,73) and DAG species with very long-chain acyl moieties (60,61). It is noteworthy that the per-

TABLE 5
Acyl-CoA Selectivity of Microsomal DGAT from Various Plant Sources

	Acyl-CoA selectivity							
Plant species	12:0	16:0	18:0	18:1	18:2	18:3	22:1	References
Maize	1			2			N	(71)
Cuphea	1			2			N	(71)
Cocoa		1	2	3				(68)
Safflower		1	2	3				(69)
Turnip-rape		1	3	2				(69)
Canola	1			2			3	(71)
Rape	1			2			3	(71)

For each sample, DGAT selectivity is ranked in descending order of activity: 1, highest; 3, lowest; N, negligible.

TABLE 6
Activators of Plant DGAT

Category	Activator	DGAT source	Maximum effect[a] (%)	References
Metabolite	ATP	*B. napus*	+240	(44,75)
	CoA	*B. napus*	+1,400	(44,75)
	G-3-P	*B. napus*	+650	(60)
	PA	*B. napus*	+200	(44,75)
Protein	ACBP	*A. thaliana*	+70	(45)
	ASP	*B. napus*	+100	(78)
	BSA	*A. thaliana*	+100	(45)
		B. napus	+500	(46)
		E. guineensis	+270	(15)
	Hemoglobin	*B. napus*	+140	(46)
	α-lactalbumin	*B. napus*	+150	(46)
Electrolyte	Mg^{2+}	*B. napus*	+2,500	(44,75)
		A. thaliana	Not reported	(45)
		S. oleracea	+200	(35)
		E. guineensis	+113	(15)
	Mn^{2+}	*S. oleracea*	+200	(35)
	F^-	*B. napus*	+230	(46)
	NaCl	*B. napus*	+140	(46)
Other	Cytosolic fractions	*B. napus*	+200	(46)
	Dithiothreitol	*E. guineensis*	+270	(15)

[a]Each value is relative to the control without the additive.
ACBP, acyl-CoA binding protein; ASP, acylation stimulating protein; G3P, *sn*-glycerol-3-phosphate.

sisting selectivity of DGAT from low–erucic acid *Brassica napus* varieties for erucoyl-CoA is consistent with the breeding efforts that modified the genes encoding enzymes that catalyzed FA elongation but not DGAT genes (71). Griffiths *et al.* (74) suggested that the strong selectivity of DGAT from common borage for γ-linolenic acid could potentially lead to the efficient removal of this FA from the acyl-CoA pool and thereby account for the rare occurrence of this FA at the *sn*-2 position of TAG, compared with the efficient DGAT-catalyzed incorporation of this FA at the *sn*-3 position.

In addition to advancing our knowledge of the factors that control the acyl composition of seed oils, the extensive studies of acyl-CoA preference of plant DGAT offer new insights into developing strategies for genetic engineering of seed oil composition. For instance, in an attempt to generate unusual FA in seed oils of certain species, one should take into account the compatibility of the DGAT in the host plants with the engineered unusual FA. In this context, activity of DGAT from *Cuphea* and maize toward erucic acid was negligible (71), whereas the incorporation of EPA (20:5*cis*$\Delta^{5,8,11,14,17}$) and DHA (22:6*cis*$\Delta^{4,7,10,13,16,19}$) into TAG via DGAT action is apparently feasible in developing flax seeds (47).

MODULATORS OF DGAT ACTIVITY

A better understanding of DGAT modulators may provide new opportunities to enhance TAG accumulation in oilseed plants. Currently known activators and inhibitors are summarized in Tables 6 and 7, respectively. A number of metabolites involved in the TAG biosynthetic pathway have been shown to enhance *B. napus* DGAT activity substantially (44,60,75). *sn*-Glycerol-3-phosphate may stimulate CPT activity and thereby facilitate the channelling of PC to DAG, which is an acyl acceptor in the DGAT-catalyzed reaction (60). Alternatively, exogenous PA has been shown to fuel the production of DAG via PA phosphatase, and thereby favor the DGAT-catalyzed reaction

TABLE 7
Inhibitors of Plant DGAT

Category	Inhibitor	DGAT source	Maximum effect[a] (%)	References
Protein	BSA	*C. tinctorius*	–97	(43)
	Soybean trypsin inhibitor	*B. napus*	–35	(46)
Electrolyte	Zn^{2+}	*A. thaliana*	Not reported	(45)
	Mg^{2+}	*C. tinctorius*	–36	(43)
	Ca^{2+}	*C. tinctorius*	–57	(43)
	Hg^{2+}	*C. tinctorius*	–76	(43)
	I^-	*B. napus*	–40	(46)
Other	Niacin	*A. thaliana*	–30	(45)
	Tannin	*B. napus*	Not reported	(132)
	2-Bromooctanoate	*B. napus*	–50	(48)

[a]Each value is relative to the control without the additive.

(44,75). Adenosine triphosphate (ATP) and CoA, which are the important key metabolites in the synthesis of acyl-CoA, the acyl donor in the DGAT-catalyzed reaction, have been shown to exhibit a stimulatory effect on *B. napus* DGAT activity (44,75). Whether or not these metabolites act as allosteric effectors of DGAT *in planta*, however, remains to be investigated.

Among various stimulating proteins (Table 6), BSA has been used routinely in DGAT assays to increase the sensitivity of enzyme detection (Table 6) (46). In studies with MD embryos of *B. napus*, BSA increased DGAT activity in a concentration-dependent manner, with a maximum of 5-fold stimulation at a concentration of 3–4 mg BSA mL^{-1} (46). The BSA-stimulatory effect on DGAT may be related to the acyl-CoA binding ability of BSA, such as prevention of acyl-CoA micelle formation (detergent effect), protection of acyl-CoA from thioesterase activities, and/or the preference of DGAT for acyl-CoA in protein-bound form (15,46,76,77). These hypotheses are supported by another observation that a recombinant acyl-CoA binding protein increased the activity of *A. thaliana* DGAT1 expressed in insect cell culture by up to 70% (45). Interestingly, BSA was found to severely inhibit the activity of safflower DGAT (43).

In another study with the DGAT from MD embryos of *B. napus* (78), a similar stimulatory effect on DGAT has been observed in the presence of human acylation stimulating protein (ASP). Transformation of *B. napus* with the cDNA encoding ASP resulted in an apparent increase in seed size and lipid content per seed in some transgenic lines (78). As well, transformation of *B. napus* with cDNA encoding ASP or BSA resulted in some lines having changes in the FA composition of the seed oil (79). Thus, ASP or BSA appears to exert modulatory effects on DGAT activity *in vivo*. It is plausible that one or more certain small organic molecules act as DGAT modulator because the activity of microsomal DGAT from MD cultures of *B. napus* was shown to be enhanced by the addition of the cytosolic fraction that lost its stimulatory effect after ashing (44). A number of electrolytes have also been shown to activate or inhibit DGAT activity (Tables 6 and 7). Observed modulatory effects brought about by exposure of enzymes to electrolytes have been attributed to changes in enzyme conformation (46).

SOLUBILIZATION AND PARTIAL PURIFICATION OF DGAT

DGAT are highly hydrophobic, membrane-bound enzymes, and thus their solubilization necessitates the use of detergents, which are known to be enzyme denaturants and inhibitors of enzyme activity (80). Nonetheless, the successful development of a solubilization scheme facilitates not only the purification process but also the removal of endogenous DAG for selectivity assays (30).

Solubilization of DGAT has been reported for the enzyme from spinach leaves (35), germinating soybean (80), developing maize embryos (30), and MD cultures of *B. napus* (46,48,81). Solubilization of DGAT from spinach leaves with salt solution favorably yielded a 10- to 40-fold purification and a recovery of 20% enzyme activity (35). Kwanyuen and Wilson (80) reported that solubilization of microsomal DGAT from germinating soybean using ionic agents such as NaCl, KCl, sodium cholate, and deoxycholate at various concentrations resulted in significant inhibition or loss of enzyme activity. Treatment of the same membrane preparation with 9 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate also reduced the activity drastically, but yielded the highest recovery of specific activity among all tested agents and produced a three-fold purification (80). In another study with microsomal DGAT from MD cultures of *B. napus* (48), ionic detergents similarly inhibited enzyme activity substantially, whereas a nonionic detergent, *n*-octyl β-D-glucopyranoside, was found to be the least inhibitory. Further studies on the effects of different nonionic detergents indicated that dispersion of the enzyme with MEGA-8 could ideally maintain the enzyme activity (81). Subsequently, the solubilization of the enzyme with MEGA-8 was optimized at a detergent (1% wt/vol) to protein ratio of 2:1, in the presence of 2 M NaCl (46). This solubilized enzyme was later used in photoaffinity labeling studies with the radiolabeled substrate analog [^{125}I]12-[(4-azidosalicyl)amino]dodecanoyl-CoA ([^{125}I]ASD-CoA), which identified a 39-kDa polypeptide in the solubilized fraction (82). The solubilization procedure for *B. napus* DGAT was reproduced using microsomal DGAT from developing maize embryos with high recovery of enzyme activity, although it was found to be ineffective in the removal of endogenous DAG (30). Valencia-Turcotte and Rodríguez-Sotres (30) proposed another method in which dried microsomal and oil body fractions were delipidated by repeated washing with benzene followed by solubilization with a solution of methanol/acetic acid/water (1:1:1, by vol) in preparation for molecular sieve chromatography using controlled-pore glass. The active fractions could subsequently be identified by assay with exogenous DAG, PC, and [^{14}C]oleoyl-CoA (30).

Partial purification of solubilized DGAT has been reported for a number of plant tissues, including germinating soybean seeds (80), developing safflower seeds (43), MD culture of *B. napus* (46,81), and spinach leaves (35). DGAT from germinating soybean cotyledon has been highly purified and the final enzyme fraction was believed to be homogenous (80), but reported later to contain oil body proteins (83). Characterization of this partially purified enzyme indicated the presence of three possible subunits (84). DGAT has been purified to apparent homogeneity from an oil body fraction of *Mortierella ramanniana* (56).

DGAT1

In 1998, there were two reports on the cloning of putative acyltransferase genes from mouse and human based on sequence homology to ACAT (85). Expression of the mouse cDNA in insect cells increased DGAT activity in the microsomes (85). The isolation of *DGAT1* cDNA from mouse was immediately followed by the identification of other *DGAT1* members from a number of plant species, including *Arabidopsis* (23,54,55,86), olive (87),

tobacco (23), oilseed rape (88), burning bush (89), castor bean (64), and tung (32). Cloning of the same *Arabidopsis* DGAT1 (*AtDGAT1*) cDNA has been reported in four independent studies (23,54,55,86). Although the *AtDGAT1* gene was obtained from expressed sequence tag (EST) or genomic clones through similarity searches of database in all three studies, Routaboul *et al.* (86) were capable of further confirming the gene identity by cloning of two mutated alleles (AS11 and ABX45) from the T-DNA insertion mutant collection by screening for wrinkled, incompletely filled seeds with reduced oil composition.

The identification of *AtDGAT1* assisted the subsequent cloning of *DGAT1* cDNAs from olive (87), tobacco (23), burning bush (89), and tung (32). The deduced amino acid sequences represented by two olive *DGAT1* cDNAs differ by 26 amino acids in the central part of the polypeptide (87), whereas a shorter oilseed rape DGAT-encoding cDNA, designated as *BnDGAT2*, is a truncated version of the *BnDGAT1* and encodes a protein lacking the hydrophilic N-terminal segment found in BnDGAT1 (88). Interestingly, the DGAT1 from burning bush exhibits both acetyl and long-chain acyltransferase activity and can probably switch between either specificity depending on *in vivo* conditions (89).

Alignment of the deduced amino acid sequences of DGAT1 from different plant species is presented in Figure 2A. In the phylogenetic tree, the plant and animal DGAT1 proteins apparently separate into two major clusters (Fig. 2B). The variants between different plant and animal DGAT1 proteins are primarily concentrated at the N-terminal segments (Fig. 2A), which might account for the differences in enzymic properties among DGAT1 from different plant sources. For instance, although the deduced amino acid sequence of DGAT1 from burning bush shares 91% similarity with the *Arabidopsis* counterpart (89), the unique acetyltransferase activity of burning bush DGAT can probably be ascribed to the greatly different N-terminal end. The divergence between DGAT and ACAT is also centered on the N-terminal parts of the proteins (23). In fact, it has been demonstrated that the N-terminal fragment of BnDGAT1 interacts with acyl-CoA exhibiting greater affinity for erucoyl-CoA than oleoyl-CoA (78,90). It is also noteworthy that the N-terminus of BnDGAT1 has been localized to the cytosolic side of the ER and shown to self-associate into tetramers (90). The N-terminus also appears to be important in the self-association of ACAT1 (91) and human DGAT1 (92).

In general, each DGAT1 protein consists of 9–10 putative transmembrane domains and a relatively hydrophilic N-terminal segment (Fig. 2A). Topological studies of tung DGAT1 suggested that both N- and C-termini of the protein are oriented toward the cytosolic side of ER membranes (32). Common functional domains predicted in several DGAT1 proteins include phosphorylation sites (55,64,88), leucine zippers (23,55,88), and DAG/phorbol-binding motif (54,87,88). An invariant proline residue is believed to participate in acyl-CoA binding (54). In addition, an inner membrane component signature of the binding-protein–dependent transport system is uniquely present in DGAT1 from castor bean (64), implicating potential interaction of the enzyme with acyl-CoA binding proteins.

DGAT2

In 2001, Lardizabal *et al.* (93) reported the cloning of two type 2 DGAT species from the oleaginous fungus *Mortierella ramanniana*. The deduced amino sequences of the two unrelated polypeptides of MrDGAT2 shared no homology with DGAT1. Cases *et al.* (94) used the *MrDGAT2* sequence for homology searches of EST libraries and successfully identified the DGAT2-related genes from mouse and human. From the studies with single and multiple gene disruptions, Sandager *et al.* (95) suggested that DGAT2 was the major TAG-synthesizing enzyme in yeast. Based on the amino acid sequence of a putative *DGAT2* homologue in *Arabidopsis* (92), a novel DGAT2 cDNA has recently been identified in tung (32). The encoded protein possesses two predicted transmembrane segments and both N- and C-termini are oriented toward the cytosolic side of ER (32).

Cases *et al.* (94) have developed assay conditions to differentiate DGAT1 and DGAT2 activity in microsomes from several mouse tissues including brain, heart, muscle, liver, and adipose tissue. A method for determining the relative contributions of DGAT1 and possible DGAT2 activity in plant microsomes, however, has not yet been developed. Thus, at this time, it is not possible to ascribe the effects of activators or inhibitors of plant DGAT activity to a specific DGAT isoform in assays conducted with microsomes. Some idea of the relative contributions of DGAT1 and DGAT2 to oil accumulation in plant tissues might be obtained using immunochemical methods with specific antibodies directed against each of the isoforms. Nykiforuk *et al.* (88) have raised monospecific polyclonal antibodies against synthetic peptides representing peptide segments within *B. napus* DGAT1.

NOVEL DGAT FAMILIES

A gene encoding a bifunctional enzyme (*AcDGAT*) exhibiting wax ester synthase and DGAT activity has been identified in *Acinetobacter calcoaceticus* and found to be unrelated to known wax synthases, DGAT1 and DGAT2 (96). The enzyme, however, was shown to share similarity to a large family of putative proteins in *Arabidopsis*, implying that the acyl-CoA–dependent synthesis of TAG in *Arabidopsis* may be accomplished by multiple enzymes (96). AcDGAT may also be a membrane-bound enzyme due to the presence of a predicted membrane-spanning region (96). However, a cytosolic DGAT was recently identified in developing peanut cotyledons and its full-length cDNA cloned (97). The deduced amino acid sequence of this novel DGAT shares 13% identity with the bifunctional AcDGAT and less than 10% identity with DGAT1 and two family members (97). The presence of orthologous genes in the genomes of other plant species implicates the possible existence of a cytosolic TAG synthetic pathway in plants that is not restricted to peanut (97).

THE ROLE OF DGAT IN TAG SYNTHESIS

The spatiotemporal expression of DGAT in plants has provided insight into the role of DGAT in regulating TAG biosynthesis.

A

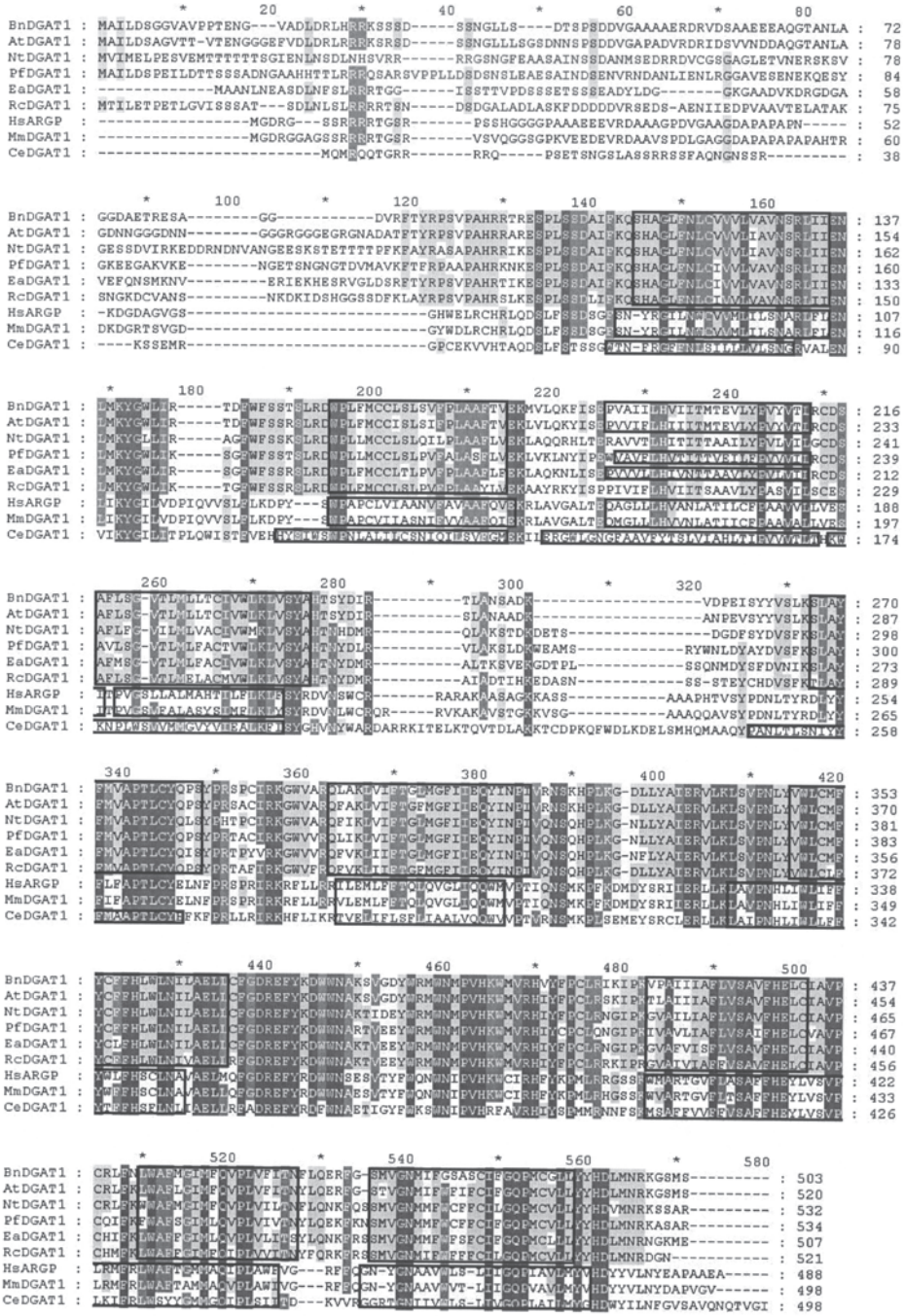

FIG. 2. Comparison of deduced amino acid sequences from different putative or demonstrated type-1 DGAT. (A) Amino acid sequence alignment. DGAT-1, diacylglycerol acyltransferase-1; ARGP, acyl-CoA:cholesterol acyltransferase-related gene product. Alignment was performed using ClustalX v1.83 formatted in Genedoc (Multiple Sequence Alignment Editor and Shading Utility v. 2.6). The residues blocked with a white foreground on a dark grey background are 100% conserved bases, residues with a white foreground on a grey background are 75% or more conserved bases, and residues aligned with a black foreground on a light grey background are 50–74% conserved bases. Putative transmembrane domains are boxed. GenBank accession numbers are shown in parentheses: *BnDGAT1*, *B. napus* (AF164434); *AtDGAT*, *A. thaliana* (AF051849); *NtDGAT*, *N. tabacum* (AF129003); *PfDGAT*, *P. frutescens* (beefsteak mint; AF298815); *EaDGAT*, *E. alatus* (winged euonymus; AY751297); *RcDGAT*, *Ricinus communis* (AY366496); *HsDGAT*, *H. sapiens* (L21934); *MmDGAT*, *M. musculus* (AF078752); *CeDGAT*, C. *elegans* (AF221132). (B) The phylogenetic tree was constructed by the neighbor joining method using the MEGA 3 program (129) with 1,000 bootstrap repetitions. The bootstrap values (%) are given at the respective nodes. (Continued)

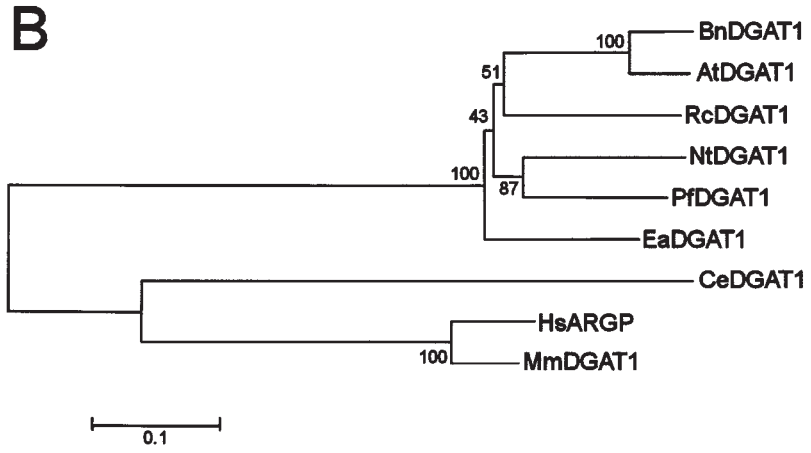

FIG. 2. (Continued)

Spatial expression of DGAT has been investigated primarily in *Arabidopsis* (41,54,55,98). In general, it has been shown that the *Arabidopsis* DGAT1 transcript and protein were at the highest concentration in oil-forming organs, specifically inflorescence and developing seeds (55,98), although considerable amounts of transcript and protein were also apparent in leaf (41,54,55), flower (41,54), root (41,54), and stem (41,98) tissues. A similar expression pattern for *DGAT1* was also observed in oilseed rape (55). Interestingly, a recent investigation on the spatiotemperal expression of tung DGAT1 and DGAT2 as well as their acyl-CoA selectivities suggested that the former type plays a constitutive role in TAG metabolism in several tissues whereas the latter type is more specific in production of TAG containing eleostearic acid in seeds (32).

Oilseed development is a three-stage process, which includes cell division, biosynthesis of storage materials, and desiccation. The temporal expression of *DGAT* in developing seeds is generally synchronized with TAG biosynthesis. For instance, *DGAT1* transcript was detectable during later stages of embryo development in olive when the expression of FA synthase dropped to an undetectable level (87). The expression of a DGAT1-β-glucuronidase (GUS) fusion protein was visualized to be most intense in maturing embryos and pollen of *Arabidopsis*, whereas the GUS activity was undetectable during early embryogenesis or in desiccating seeds (98). Earlier studies showed that high DGAT activity in developing seeds was parallel to the active phase of rapid lipid accumulation and subsequently reduced as the seed lipid content reached a plateau (47,65,73,99).

As DAG is located at the branch point between TAG and membrane phospholipid biosyntheses (Fig. 1), DGAT potentially plays a decisive role in regulating the formation of TAG in the glycerolipid synthesis pathway. Considering the putative protein kinase-targeting motif conserved between DGAT and other acyltransferases, it is believed that at least some of the regulation operates at the protein level (54,100). Some characteristic regulatory elements have also been recognized in the *AtDGAT1* gene and may be important in regulating the expression of the gene during seed maturation (23,98), although it has been suggested that DGAT activity in oilseed rape is not tightly regulated in relation to transcript (100,101). The regulatory role of DGAT is supported by multiple lines of evidence that have suggested that modulation of DGAT activity results in sizable effect on seed oil production. For instance, mutation of the *DGAT1* gene severely affects seed development in *Arabidopsis*, including the appearance of wrinkled and incompletely filled seeds (86), reduction in seed oil content (54,86,102) and increased DAG/TAG ratio (50), increased flow of elevated FA toward β-oxidation (102), and aberrant seedling growth (86). Whereas the effect of osmoticum on enhancing TAG formation has been noticed in several plant species (103–107), the induction of TAG accumulation in oilseed rape by sucrose has been correlated with the increase in DGAT activity (88,101,108, 109). Similarly, modulation of lipid accumulation in oilseed rape by light/dark treatments indicated that the activity of DGAT was the lowest among the four enzymes in the Kennedy pathway, and such modification of carbon flux resulted in changes in the DAG pool but not other intermediates (53). In other studies, the rate-limiting role of DGAT in TAG formation has also been proposed from the general observation of the lowest specific activity of DGAT compared with other Kennedy pathway enzymes (18) and the highest accumulation of DAG as a Kennedy pathway intermediate (110,111), although it should be noted that DAG accumulation can also be due to a shortage of acyl-CoA (112). Interestingly, it has also been suggested that the rate-limiting role of DGAT might also be important for the equilibration of DAG with the PC pools to achieve some degree of polyunsaturation of FA moieties at the level of PC (68,113). In this context, the most convincing observation is that methanesulfonate-induced mutation of *AtDGAT1* resulted in substantial increase in the proportion of PUFA (e.g., linoleic and α-linolenic acids) to monosaturated FA (e.g., oleic and erucic acids) in *Arabidopsis* (50).

Despite the remarkable effect of plant DGAT on TAG accumulation, the rate-limiting role of DGAT is not observed in all plant species, as exemplified by the independence of TAG production in lupin species from DGAT activity (114). As suggested by Bao and Ohlrogge (112), oil content is a quantitatively inherited trait and is, therefore, probably regulated by multiple steps involving a number of enzymes including FA synthases, elongases, and hydrolases. Nonetheless, overexpression of DGAT genes is a promising strategy to boost TAG yield. Whereas overexpression of *AtDGAT1* in tobacco leaves produced up to 7-fold increase in TAG content of leaf tissues (23), seed-specific expression of the same cDNA in *Arabidopsis* led to an overall increase of 11–28% in seed oil content (115).

THE ROLE OF DGAT IN SEED GERMINATION

As stored TAG in seeds is broken down actively to meet the high energy requirement for seedling development during germination, it was surprising to observe that the expression of *AtDGAT1* was up-regulated in germinating seeds and young seedlings (54). The DGAT expression in seedlings was later confirmed in transformation studies with a DGAT-GUS fusion construct (98). The increased sensitivity of *Arabidopsis* DGAT mutants to sugars and various stress conditions during germination and seedling development strongly suggests that DGAT also plays a post-embryonic role in plants (116). Although it has been known that germinating seedlings are capable of TAG synthesis (117), Lu *et al.* (98) proposed that DGAT might also play a role in sequestration of TAG-released FA and coordination of metabolism during seedling development in response to environmental cues.

Recently, He *et al.* (118) also proposed that TAG synthesis in early seedling development may be of relevance to heading off the osmotic effects of excessive sucrose, which is the major breakdown product of storage oil during germination. In their studies, lipid synthesis in germinating castor seed cotyledons was shown to correlate with *de novo* expression of *RcDGAT1,* and such process is probably regulated by proteolytic degradation (118).

THE ROLE OF DGAT IN LEAF METABOLISM

During protoplast isolation from *Arabidopsis* leaves, Browse *et al.* (119) reported that the mass of all major polar lipids decreased by 10–20% while the amount of neutral lipids including TAG increased by three-fold compared with the original leaf tissue, concomitant with the transfer of hexadecatrienoic acid (16:3), a characteristic acyl component of thylakoid monogalacyosyldiacylglycerol, to other lipid classes. From similar observations of acyl migration from polar lipids to neutral lipids in ozone-treated spinach leaves, Sakaki *et al.* (120,121) proposed that free FA were released from monogalacyosyldiacylglycerol by galactolipase, esterified to acyl-CoA by the catalytic action of acyl-CoA synthetase, and eventually acylated with *sn*-1,2-DAG to form TAG in a DGAT-catalyzed reaction. A recent study has shown that the expression of *DGAT1* was up-regulated during leaf senescence in *Arabidopsis* and the accumulated TAG was similarly enriched in hexadecatrienoic acid and linolenic acid (41). Although the significance of the sequestration of galactolipid FA in TAG remains unknown, some authors have suggested that it is a means to diminish the toxicity of free FA (120) or an intermediate step in the conversion of thylakoid FA to phloem-mobile sucrose in response to stresses (41). Similar results were obtained when leaf tissues were incubated in 0.5 M sorbitol solution (119), implying that such a conversion of polar lipids to neutral lipids may be a response to stresses.

ACYL-COA–INDEPENDENT PATHWAYS FOR TAG FORMATION

The *Arabidopsis* mutants (ABX45 and AS11) possess a mutated allele of *AtDGAT1*, which was proven to be single-copy gene by Southern blot analyses (54,86). The inactivation of *AtDGAT1* gene does not completely abolish the TAG-synthesizing capacity of the plants (54,86,98), suggesting the existence of other DGAT isoforms and/or non–DGAT-catalyzed reaction(s) that could lead to TAG formation in plants. Two enzyme activities (i.e., PDAT and DGTA) catalyzing acyl-CoA–independent synthesis of TAG have been described.

PDAT

PDAT is a transacylase that catalyzes the acyl-CoA–independent transfer of FA from phospholipid to *sn*-1,2-DAG to form TAG and lyso-phospholipid. The PDAT-encoding gene identified in yeast shares sequence homology to mammalian lecithin-cholesterol acyltransferase (EC 2.3.1.43), which catalyzes the acyl transfer from lecithin to cholesterol (6). The PDAT-catalyzed reaction has been shown to mediate the majority of TAG formation in yeast during exponential growth, whereas mutation of the four yeast DGAT-like genes has been shown to result in little or no effect on TAG synthesis (6). Concomitantly, a significant increase in TAG content in PDAT-overexpressing yeast cells was observed (122). Dahlqvist *et al.* (122) characterized PDAT in microsomal preparations from developing seeds of sunflower, castor bean, and *Crepis palaestina*, in addition to the cloning of the yeast PDAT-encoding gene. The specificity of PDAT from different plant sources toward acyl donors is species-dependent (122,123), such that the *C. palaestina* PDAT preferentially catalyzes the transfer of the vernoloyl groups (*cis*-12-epoxyoctadeca-*cis*-9-enoic acid) of phospholipids into TAG, whereas castor bean PDAT catalyzes the incorporation of both ricinoleoyl (HO-18:1) and vernoloyl groups (122). PDAT in oilseed plants may therefore play a critical role in removal of unusual FA from membrane phospholipids by the selective transfer of these FA into TAG (122). The substrate specificity of yeast PDAT depends on the head group of the acyl donor, the acyl group transferred, and the acyl chains of the acceptor DAG molecule (122). Recently, a PDAT-encoding cDNA that shares 28% amino acid identity to the yeast PDAT has been identified in *Arabidopsis* (124), suggest-

ing that PDAT activity is not limited to the unusual FA-producing oilseed plants. Gene identity was confirmed by overexpression in *Arabidopsis* and the protein was found to exhibit broad substrate specificity toward different phospholipids as acyl donor containing different acyl moieties ranging from C10 to C22 (124). Despite the 10-fold increase in PDAT activity of the transformants, no alteration in FA composition in either vegetative parts or in the oil accumulating seeds was observed, implying that PDAT might not be important for the control of lipid composition or redistribution of acyl groups between lipids in *Arabidopsis* (124). A recent study with a knockout mutant of *Arabidopsis* possessing a T-DNA insertion in the PDAT locus indicated no significant changes in FA content and composition in the mutant seeds (125). Although it is plausible that PDAT may not be major TAG contributor in plants, the specific physiological roles of PDAT remain to be elucidated.

DGTA

In 1993, Lehner and Kuksis (5) first reported the purification and characterization of a DGTA from rat intestinal microsomes. The purified enzyme did not catalyze the incorporation of radioactivity from [1-^{14}C]oleoyl-CoA into any acyl acceptors, but catalyzed a transacylation reaction in which the free hydroxyl group of a DAG molecule was acylated with a FA moiety from another DAG molecule, forming a MAG and a TAG molecule. The enzyme was found to utilize both *sn*-1,2-DAG and *sn*-2,3-DAG at similar rates but not *sn*-1,3-DAG or other neutral lipid esters (5). DGTA also appears to catalyze the acyl transfer from a TAG molecule to a MAG molecule to yield two DAG molecules in the reverse reaction, which may be important in further desaturation of acyl groups from TAG via the interconversion between DAG and PC (52). By manipulating the Mg^{2+}-dependent conversion of PA to DAG in microsomal membrane preparations from developing sunflower seeds, the flux of TAG formation from DAG in the presence of Mg^{2+} after EDTA treatment was found to be primarily independent of acyl-CoA, suggesting the more important role of DGTA and other acyl-CoA–independent pathways for TAG synthesis in this crop (126). Mancha and Stymne (127) observed a remodeling of *in situ*–synthesized TAG species in microsomal preparations from developing castor bean endosperm, such that ricinoleate in TAG was substituted by non-hydroxylated FA groups derived from polar lipids and mainly from PC, implicating the activity of DGTA. To date, however, no cDNA encoding DGTA has been isolated from any source. Recently, Stymne *et al.* (128) showed that yeast PDAT also displayed transacylase activity, which may have accounted for the observation on apparent DGTA activity in plants (52).

CONCLUSIONS AND FUTURE DIRECTIONS

In general, oilseed biotechnology sets out to accomplish two ultimate goals: (a) to increase oil accumulation in the seed and (b) to produce a desirable FA composition in the seed oil. A detailed understanding of the terminal step in TAG formation in plants will provide valuable knowledge for accomplishing these long-term goals. The physiological role of DGAT in regulating TAG accumulation in plants opens a new perspective on improving seed oil content by overexpression of DGAT-encoding cDNA in transgenic plants. Although the results from *AtDGAT1*-overexpressing tobacco and *Arabidopsis* have been promising, the feasibility of this approach for improving major oilseed crops remains to be investigated. The biochemical properties of DGAT vary among different plant species as well as a number of environmental conditions. cDNA encoding plant DGAT1 and DGAT2 have been functionally expressed in yeast. The recent discovery of a bifunctional DGAT/wax ester synthase gene from *Acinetobacter calcoaceticus* (96) and a novel cytosolic DGAT from developing DGAT cotyledons (97) may lead to the identification of orthologues of these novel DGAT from other species. The identification of all DGAT-encoding genes will also assist in investigation of the physiological role(s) of DGAT in plants through the use of multiple knockout mutants. In *Arabidopsis*, the increase in the proportion of PUFA to monounsaturated FA that resulted from methanesulfonate-induced mutation of *AtDGAT1* (50) implicates the importance of DGAT in influencing FA composition of the seed oil. Thus, site-directed mutagenesis of *DGAT1* might create enzyme variants with altered FA selectivity properties. In this context, the deduced amino acid sequence of the currently known plant DGAT implicates that the difference in enzymic properties among different plant DGAT may be primarily dependent on the variation in N-terminal regions of the proteins. Investigations of DGAT modulators could lead to other strategies for enhancing seed oil content. Whilst the enhancement of oil deposition associated with increased seed size in ASP-overexpressing oilseed rape is encouraging (79), a comprehensive study of potential interacting partners of DGAT within the oil-forming cell might lead to additional strategies for altering seed oil content.

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Cyclic Fluctuations of 3-Hydroxy-3-methylglutaryl-CoA Reductase in Aortic Smooth Muscle Cell Cultures

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ABSTACT: The cyclic fluctuations of HMG-CoA reductase activity and mRNA are reportedly related to feeding the cells in culture or to variations in food consumption by the animals over a 24-h cycle. In this work, we demonstrate cyclic increments in HMG-CoA reductase activity in smooth muscle cells (SMC) not associated with the culture feeding. Since reductase activity also shows a marked rise preceding the S phase, one of the major goals of the present work was to evaluate this dual role of reductase activity and mRNA fluctuations related to the cell cycle and to food intake in the SMC-C/SMC-Ch cultures derived from control-fed (SMC-C) and cholesterol-fed (SMC-Ch) chicks. The period and amplitude oscillations in HMG-CoA reductase activity varied depending on culture conditions: lipoprotein-deficient serum vs. FBS, young vs. senescent cells, or confluent vs. nonconfluent cultures. The HMG-CoA reductase mRNA concentration showed a marked rise after feeding not correlated to the fluctuation activity, suggesting posttranscriptional modulation. Reductase activity and mRNA were down-regulated in SMC-Ch. Since the nutritional culture conditions were the same in both cell lines, these findings indicate that consumption of a high-cholesterol diet by the animals prior to the establishment of the SMC cultures induced changes in the HMG-CoA reductase gene expression in aortic SMC.

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The enzyme HMG-CoA reductase (EC 1.1.1.34), from the endoplasmic reticulum, catalyzes the conversion of HMG-CoA to mevalonate, the rate-limiting step in cholesterol biosynthesis (1). The rate of cholesterol biosynthesis from different tissues was shown to have a diurnal rhythm many years ago (2–4). These rhythmic changes are associated with changes in HMG-CoA reductase activity (1,2,5,6). In rodent liver, diurnal variation of hepatic cholesterol synthesis is driven primarily by varying the steady-state mRNA levels for HMG-CoA reductase (6–11). The diurnal rhythm of HMG-CoA reductase from chick liver and intestine has also been observed in our laboratory. In this animal, both reductase activities were maximal during the light period, in agreement with chick feeding habits. However, chick liver and intestine reductase do not show diurnal rhythm at hatching (5) or during the first week of life, when the chick is still reabsorbing the yolk (12) and thereby developing a transient hypercholesterolemia, which becomes almost three times higher in cholesterol-fed chicks (13). Indeed, the rise in reductase activity is depressed in hypercholesterolemic chicks (13) and following cholesterol or mevalonate feeding in cell cultures (14). Moreover, the diurnal rhythm of mevalonate metabolism by sterol and nonsterol pathways and of mevalonate-activating enzymes has been demonstrated (15). The multivalent feedback regulation of HMG-CoA reductase involves cholesterol and one or more of the other products that are synthesized from mevalonate (16). Both cholesterol and mevalonate are required for the depression of reductase activity at the transcriptional, posttranscriptional, and posttranslational levels (17–20). The diurnal variation in activity is a good example of short-term physiological regulation of hepatic HMG-CoA reductase (1,2) and is due to changes in the state of phosphorylation of the enzyme; however it has been reported that the diurnal variation in hepatic HMG-CoA reductase is due to changes in enzyme protein levels rather than changes in the phosphorylation state of the enzyme (21). It has also been demonstrated *in vivo* that the short-term changes in enzyme activity are due to changes in the quantity of enzyme rather than to a modulation of the catalytic activity (22).

On the other hand, HMG-CoA reductase activity regulation participates in cell division (23–26), as reflected by a marked rise in HMG-CoA reductase activity just preceding the S phase of the cell cycle (25). However, parallel changes in HMG-CoA reductase activity have been observed in synchronized and unsynchronized cells (14). In fact, HMG-CoA reductase activity was low at the time of the medium change and increased 10- to 20-fold 5–10 h after medium change, returning to basal levels by 24 h in both synchronized and unsynchronized cells. Thus, other factors besides the cell cycle may also play a role. If the rise in HMG-CoA reductase activity is blocked by a competitive inhibitor such as compactin, the cells will not enter the S phase (23). The arrest in the cell cycle cannot be overcome by the addition of cholesterol, but rather by the addition of mevalonate (24,25). Studies in a rat embryo fibroblast cell line, synchronized by double thymidine (dThd) block and cultured in cholesterol-containing medium, have demonstrated cyclic variations of HMG-CoA reductase activity with two maxima in the S and G_2M phases (26). In this culture model, the cyclic varia-

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Abbreviations: dNTP, deoxy nucleotide 5′-triphosphate; dThd, double thymidine; LPDS, lipoprotein-deficient serum; PI, propidium iodide; RT-PCR, reverse transcription-polymerase chain reaction; SMC, smooth muscle cell; SMC-C, smooth muscle cell–control fed chicks; SMC-Ch, smooth muscle cell–cholesterol fed chicks; SRE, sterol regulatory element; SREBP sterol regulatory element-binding protein.

tions of the enzyme activities appeared to be due more to increased synthesis at given times of the cycle than to periodic dephosphorylation.

Although it has been well established that the diurnal rhythm of hepatic HMG-CoA reductase is associated with variations in food consumption over a 24-h cycle in the whole animal (5,6,8,27,28), the marked change that the reductase activity shows in cell cultures is not so well established and cannot be explained by the contribution of nutrients. This fluctuation of reductase in cell cultures has been associated with feeding the cells or with the cell cycle and DNA replication. Early experiments (27) demonstrated a small increase of the reductase activity in cultured fibroblasts 19 h after changing to fresh media supplemented with FBS, whereas a large (fivefold) increase occurred in reductase activity after changing to fresh media supplemented with lipoprotein-deficient serum (LPDS). Moreover, early experiments (14) also demonstrated that HMG-CoA reductase activity increased in a murine macrophage-like cell line (J774) under normal culture conditions (in the presence of 10% FBS) and that this variation was associated with feeding the cells. These authors also demonstrated the increase of reductase activity in confluent and unsynchronized cultures, emphasizing that other exogenous factors besides the cell cycle could play a role. On the other hand, several investigators have observed that HMG-CoA reductase activity is high in rapidly dividing cells but declines as cells reach confluence (28,29). This view contrasts with the results reported previously (14) since these authors reported that reductase activity increased in confluent cells where no transient changes in DNA synthesis or in cell number were observed. Also, marked fluctuations during the various phases of the cell cycle have been reported in HMG-CoA reductase activity, in the G_1 phase when quiescent, growth-inhibited cells are stimulated by replenishment of growth factors or serum in synchronized cultures (29), increasing to twice that of the unsynchronized cells examined concomitantly.

Thus, the cyclic fluctuations of HMG-CoA reductase activity and mRNA levels are related to feeding the cells in culture or variations in food consumption over a 24-h cycle in the animals. Since HMG-CoA reductase activity also shows a marked rise just preceding the S phase of the cell cycle, one of the major objectives of the present work was to evaluate the variations in HMG-CoA reductase activity and mRNA concentration in an *in vivo/in vitro* model of arterial smooth muscle cell (SMC) cultures and to investigate the relationships between HMG-CoA reductase, food availability, and cell division. In our laboratory, we isolated SMC from cholesterol-fed chicks (SMC-Ch), which are very proliferative in culture compared with SMC isolated from control-fed chicks (SMC-C). Thus, DNA synthesis in the S phase was fourfold higher; moreover, after 20 d of culture, SMC-Ch cells increased their cholesterol content to double that of SMC-C, producing SMC-Ch cultures under conditions mimicking a very early atherosclerosis model at the SMC level (30). We have studied cholesterol synthesis and HMG-CoA mRNA concentrations in these cultures and showed great differences between SMC-C and SMC-Ch (31,32). In this study, we demonstrate the existence of cyclic fluctuations of HMG-CoA reductase activity in nonsynchronized SMC cultures that are not correlated to the cultured feeding or to the increase of mRNA, suggesting the existence of posttranscriptional modulation of the HMG-CoA reductase and of relationships between HMG-CoA reductase activity and cell division.

MATERIALS AND METHODS

Animals. Newly hatched White Leghorn male chickens (*Gallus domesticus*) were provided by a commercial hatchery and fed *ad libitum* in a chamber with a light cycle from 0900 to 2100 hours and controlled temperatures of 29–31°C. Two groups of chicks that were 10 d old were used. The control group was kept on a standard diet (Sanders A-00) whereas the treated, or diet-group, was fed on the same diet supplemented with 5% w/w powdered cholesterol (pure grade; Panreac Química, Spain) mixed homogeneously. The diet was started at hatching and kept on until the chicks were killed (10 d after). Water was available at all times. None of the chicks died a natural death during the treatment nor developed any illness.

SMC cultures. SMC were isolated from the aortic arch of the chicks as described before (33,34) with slight modifications (30) and cultured in DMEM containing D-glucose (4.5 g/L) and L-glutamate (584 g/L) and supplemented with 1% of antibiotic cocktail composed of penicillin (100 μg/mL) and amphotericin (0.25 μg/mL) (Sigma Chemical Co.) and 10% (vol/vol) of whole serum (FBS). When specified, delipidized serum (LPDS) was substituted for whole serum during the incubation period. Medium was buffered with bicarbonate, and cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Medium was renewed every 48 h, and cells were passaged by trypsinization (0.05% trypsin–0.02% EDTA in PBS). All experiments were conducted using 3 or 4 passages (young cultures) or 13–15 passages (senescent cultures). Cells were seeded at approximately 2×10^4 cells per dish (60 mm) containing 4 mL fresh medium.

Cells were identified as vascular SMC by their "hill-and-valley" pattern of confluence (34) and by positive staining to smooth muscle actin and myosin (35). Cell number and viability were checked using the trypan blue exclusion test (Sigma). Cell viability was usually about 90%.

For culture, exponentially growing cells from three passages were synchronized by double-excess thymidine block (36). After 15 h in medium containing 2 mM thymidine, the cultures were washed two times with PBS saline solution and incubated again in normal medium for 8, 9, or 10 h. Synchronization was achieved by a second thymidine treatment for 15 h. At the end of this thymidine block, cells were carefully washed three times with 3 mL of PBS, and normal fresh medium was added. At all time points, cells were examined by light microscopy and monitored for viability by determining trypan blue exclusion. Cell viability was about 90%, and the senescent aspect of the cultures follows the normal evolution through 15 passages.

LPDS was prepared from the plasma of standard diet-fed

chickens, using a single spin gradient density ultracentrifugation according to Poumay and Ronveaux-Dupal (37).

Flow cytometry. Cell fixation was carried out as described previously (38). The cells were harvested and washed with PBS containing 10% FBS. Cell pellets were resuspended in 250 µL PBS, followed by 250 µL PBS containing 2% paraformaldehyde. After incubation for 15 min at 4°C, cells were washed, resuspended in 5 mL of ice-cold 70% ethanol, and refrigerated overnight at –20°C. Then, cells were processed for cellular DNA content and cell-cycle distribution of SMC populations. Fixed cells were resuspended in PBS containing 200 µg/mL ribonuclease and 5 µg/mL propidium iodide (PI) and then incubated for 30 min at room temperature. Cells were analyzed for red (PI, allowing DNA quantification) fluorescence using a dual laser flow cytometry FACS Vantage (Becton Dickinson Immunocytometry System, San José, CA.), with a Coherent Enterprise laser (160 mW, 488 nm and 60 mW, UV). The laser was regulated to 488 nm and 15 mW with the sample in the detector, FL_2-regulated to 495 nm (lineal), with a filter 585/42 BP. Five thousand cells were analyzed (300 cells/s); data were processed on the CellFiT (v. 2.01.2 Becton Dickinson).

Assay of HMG-CoA reductase activity in SMC cultures. HMG CoA reductase activity was measured essentially as described by Goldstein *et al*. (39) but adapted to SMC as described below. After three washings of a cell monolayer (1×10^6 cells) with PBS, cells were scraped with a rubber policeman, pelleted at $400 \times g$ then disrupted by incubation in buffer A (5 mM EDTA, 200 mM KCl, 5 mM DTT, 0.25% Brij 97, and 50 mM phosphate buffer pH 7.4) for 15 min at 37°C. After centrifugation at $10,000 \times g$ for 10 min, the protein concentration in the supernatant was determined according to Bradford (40), and about 60–100 µg of protein was used for assay of HMG-CoA reductase activity. The $10,000 \times g$ supernatant was preincubated for 5 min at 37°C in the presence of an NADPH-generating system in buffer B (12 mM DTT, 60 mM glucose-6-phosphate, 7.5 mM NADP, 20 µg/mL glucose-6-phosphate dehydrogenase, and 300 mM phosphate buffer pH 7.4), then incubated with 40 µM [3-^{14}C]HMG-CoA (specific activity: 5550 dpm/nmol) for 15 min in a final volume of 30 µL. The reaction was stopped by adding 5 µL of 5 M HCl and 20 µL of a solution containing 50 mg/mL of mevalonolactone, which acts as a marker for visualization on the TLC sheets.

Samples were then incubated for 30 min at 37°C to permit mevalonic acid to lactonize. The protein-free supernatant solution was chromatographed on a thin layer plate of silica gel G and developed in benzene/acetone (1:1, vol/vol). The plates were then placed in an iodine chamber and the mevalonolactone band (R_f 0.4–0.6) was scraped off and transferred to scintillation vials for measurement of radioactivity. Reductase activity was expressed as pmol of mevalonic acid synthesized/min/mg protein.

Data analysis. Data are expressed as mean ± SEM. Results were analyzed by Student's *t*-test.

Competitive reverse transcription-polymerase chain reaction (c-RT-PCR). Cloning was carried out in *Escherichia coli* strain JM101 (F traD36 proA+ proB+ lacI lacZΔM15/supE thi Δ(lac-proAB)) using the pGEM-T vector system I (Promega, Madison, WI). *Escherichia coli* was cultured in Luria–Bertani broth containing the appropriate antibiotic (ampicillin, 50 µg/mL). Oligonucleotide primers were synthesized with Gibco BRL (Barcelona, Spain) custom primers.

Total cytoplasmic RNA from both the SMC-C and SMC-Ch culture lines was isolated by the acid guanidinium isothiocyanate method as described elsewhere (41,42) with the slight modifications described previously (32).

The construction and sequencing of a chick HMG-CoA reductase cDNA (complementary DNA) fragment was carried out as described by Carazo *et al*. (32). This gave a fragment without primers (22 pb) of 199 pb, which was gel purified; both strands were sequenced by the chain-termination DNA sequencing method of Sanger *et al*. (43) using AmpliTaq, FS and deoxy nucleotide 5´-triphosphates (dNTP) (ABI Prism™ fluorescence labeled; PerkinElmer/Applied Biosystems Division, Foster City, CA) in a Dye Terminator Cycle Sequencing Ready Reaction (PerkinElmer/Applied Biosystems Division). The design and synthesis of chick HMG-CoA reductase homologous primers was carried out as described by Carazo *et al*. (32). To construct a chick HMG-CoA reductase mRNA standard (competitive template), we designed composite primers as described by Carazo *et al*. (32).

RT-PCR was carried out in a single reaction tube using Tth polymerase (GeneCraft GmbH, Münster, Germany), a thermostable DNA polymerase that shows RT activity, by using Mn^{2+} (44,45). All RT and PCR reactions took place in a DNA Thermal Cycler 480 (Techne, Princeton, NJ). The RT reaction was set up in 20 µL containing the appropriate amount of total cytoplasmic RNA; 10× RT buffer [670 mM Tris-HCl, pH 8.8, 166 mM $(NH_4)_2SO_4$, 0.1% Tween-20]; 1 mM $MnCl_2$; 0.25 mM dNTP; 1.25 pmol/µL downstream primer; and 0.12 units/µL of Tth polymerase and then incubated at 72°C for 30 min. Competitor DNA, as indicated above, was added before amplification. For the amplification reaction, 30 µL of a mix containing 5× PCR buffer [33.5 mM Tris-HCl, pH 8.8, 83 mM $(NH_4)_2SO_4$, 3.75 mM EGTA, 25% glycerol, 0.196 Tween-20], 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 pm/µL of both primers (upstream and downstream), and 0.05 units/µL Tth polymerase were added per reaction. Thirty cycles were routinely performed with 1 min denaturation at 95°C, 1 min annealing at 52°C, and 1 min per cycle of PCR at 72°C.

Separation and quantification of PCR products. PCR products were loaded directly onto a 2% agarose gel and electrophoresed at 5 V/cm for 1.5 or 2 h. DNA was visualized and photographed under UV light (320 nm) after ethidium bromide staining (45). The picture was digitized and analyzed using the QuantiScan Program (Biosoft, Milltown, NJ). This DNA quantification system is very sensitive and is able to detect nanogram amounts of DNA. Reactions containing the different masses of DNA were performed in triplicate. All the ethidium bromide colorimetric data included fall into the range where the DNA quantity ratio was linear. A plot of the log of the ratio of target to competitor product against the log of the competitor concentration should give a straight line with a slope of –1 (46).

Competitive RT-PCR analysis involves titrating the standard with a constant amount of sample SMC cytoplasmic RNA per reaction tube (25 ng from young cells and 50 ng from senescent cells). The amount of competitor used at which the ratio of target to competitor PCR products is equal to unity (log ratio = 0; the equivalence point) can be used to calculate the initial number of molecules in the target sequence.

RESULTS

Cell cycle analysis. We prepared unsynchronized SMC cultures of different passage numbers: early-passage cultures or young (3 or 4 passages) with exponentially growing cells or confluent cultures, late-passages cultures or senescent (13–15 passages), and synchronized cultures. Flow cytometry was used to analyze the cell cycle by cell DNA content in SMC-C cultures. Figure 1 shows the cell cycle of three passages of exponentially growing cells from unsynchronized cultures (50–60% of confluence) and the cell cycles of cultures with 100% confluency. Both in exponential growth and at confluency, the proportion of the cells in S phase was the same (almost 30%). Exponential growth SMC cultures show 50% of the cells in G_0/G_1 phase and 30% in G_2/M phase; however, at confluence in the SMC cultures the proportion of the cells in the G_0/G_1 phase is higher (70%) and in G_2/M phase is lower (10%) (Fig. 1).

Cell-synchrony experiments were performed by double excess dThd block after previous determination of the thymidine concentration range (0–4 mM) that was nontoxic for the cells. Figure 2 shows the cell cycle of three passages in exponentially growing SMC cultures synchronized by dTHd block at different times of incubation in normal medium between the first and the second 15-h dThd block. The cells were 80% blocked by thymidine at the G_0/G_1 phase with an interblockage period of 8–9 h (Fig. 2). When released from thymidine block, 20% of the cells entered the S phase, followed by less than 10% of the cells entering the G_2/M phase. Slight differences were found after 10 h of interblockage time. All experiments carried out with synchronized cultures were performed within 9 h of interblockage.

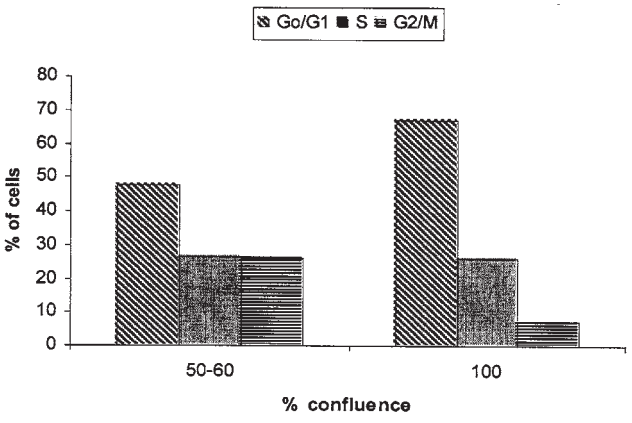

FIG. 1. Cell cycle of third passage smooth muscle cell–control fed (SMC-C) cultures at different confluence. Cells were fixed in ethanol and stained with propidium iodide prior to DNA content analysis by flow cytometry.

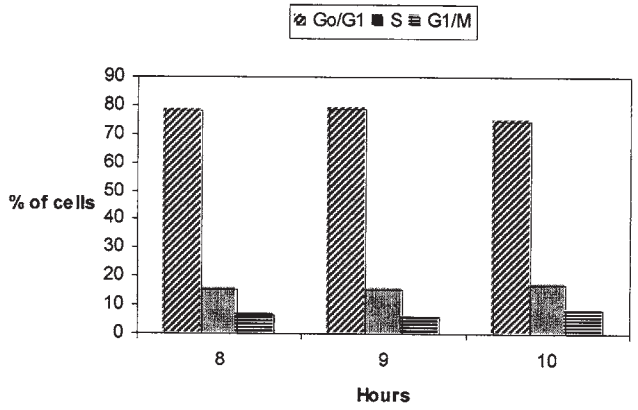

FIG. 2. Cell cycle of synchronized SMC-C cultures. Three passage cultures were grown in DMEM with 10% FBS and synchronized by the double double thymidine (dThd) block procedure with 8, 9, and 10 h of interblockage. Four hours after the second block was released, cells were fixed in ethanol and stained with propidium iodide prior to DNA content analysis by flow cytometry. For other abbreviation see Figure 1.

Effect of medium change on HMG-CoA reductase activity. Control SMC (SMC-C) cultures isolated from control chicks were prepared and at the third passage were assayed for HMG-CoA reductase activity. SMC-C shows cyclic fluctuations in HMG-CoA reductase activity although the period and amplitude of oscillations vary depending on culture conditions (LPDS or FBS, young or senescent, synchronized or unsynchronized). Figure 3 represents HMG-CoA reductase activity measured at different times during 72 h after the medium change (time zero) in synchronized cultures with FBS (A), unsynchronized cultures with FBS (B), unsynchronized cultures with LPDS (C), and 15 passages of unsynchronized cultures with FBS (D). Figure 3A shows the fluctuations of HMG-CoA reductase activity in synchronized cultures decreasing at the time of the medium change followed by a 50% increase in activity at 40 h and more than 50% at 72 h. Figure 3B shows that unsynchronized SMC-C cultures with FBS present a peak of HMG-CoA reductase activity after 5 h of the medium change (increasing around 30%). This first peak is followed by two more peaks at 32 and 64 h of around 30% of reductase activity. However, in the presence of LPDS (Fig. 3C), the absence of cholesterol produces a great induction of the reductase activity after 58 h of media change. HMG-CoA reductase activity measured in 15-passage cultures (Fig. 3D) shows the first peak 16 h after the medium change, followed by very low activity afterward that was minimal at 72 h.

Changes on HMG-CoA reductase activity in SMC-Ch and SMC-C cultures. SMC-C cultures and SMC cultures from cholesterol-fed chicks (SMC-Ch) at the third passage were assayed for HMG-CoA reductase activity. Table 1 shows HMG-CoA reductase activity measured at different times after the medium change for 12 h, reflecting first that the reductase activity was higher in SMC-C than in SMC-Ch at each time studied. Moreover, Table 1 shows the rise in reductase activity at the time of

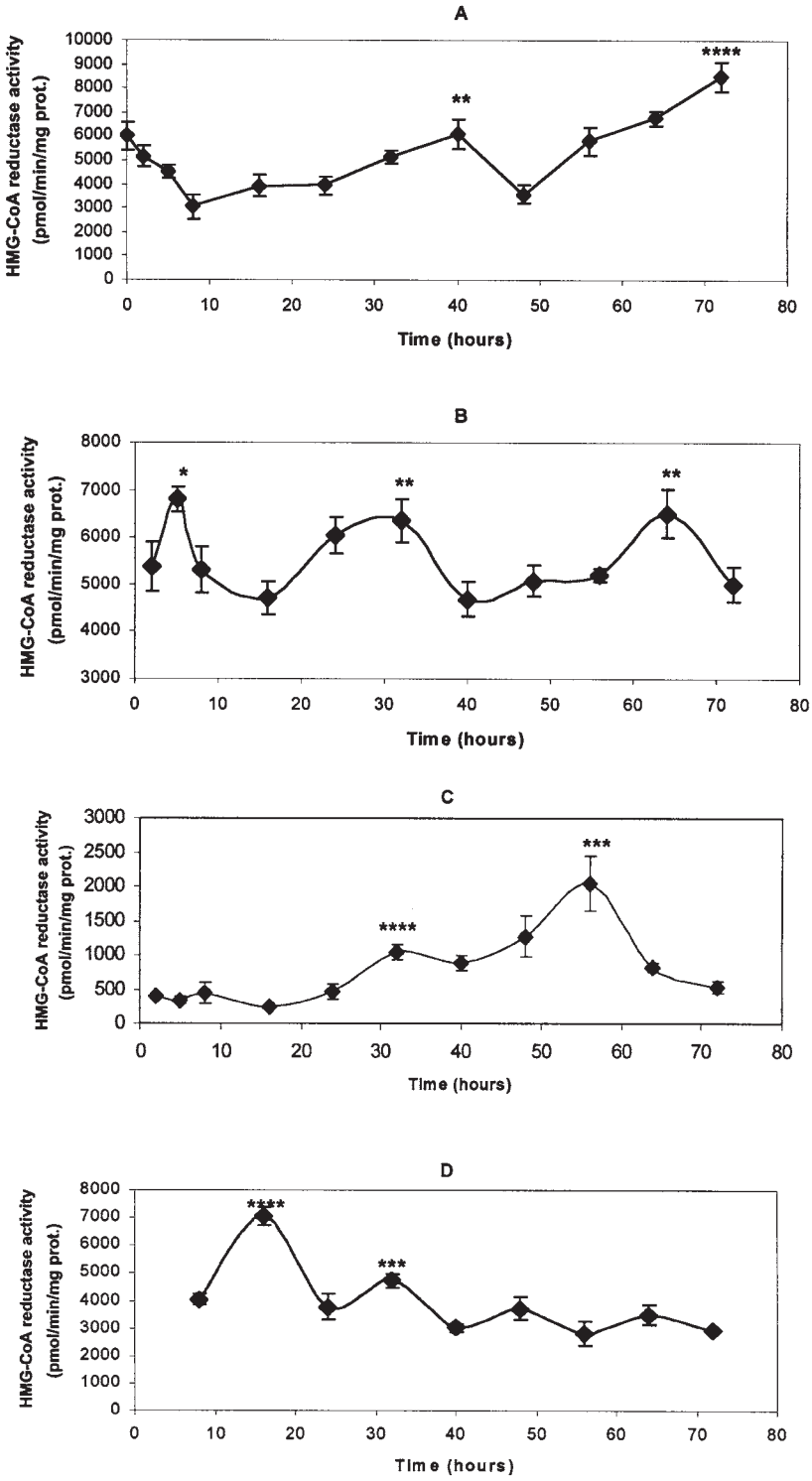

FIG. 3. Fluctuations in HMG-CoA reductase activity of three passages SMC-C synchronized cultures after media change (time 0) supplemented with FBS (A), unsynchronized SMC-C cultures supplemented with FBS (B), unsynchronized SMC-C cultures supplemented with lipoprotein-deficient serum (LPDS) (C), and 15 passages unsynchronized SMC-C cultures supplemented with FBS (D). At time zero, cultures that presented 50–60% confluence were washed with PBS and then fed fresh media. Cells were harvested at each time point, and the HMG-CoA reductase activity was determined. Results are mean ± SEM of 3 experiments. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$ vs. the previous lower value. For other abbreviation see Figure 1.

TABLE 1
HMG-CoA Reductase Activity in Aortic Smooth Muscle Cell (SMC) Cultures from Control Fed Chicks (C-SMC) and Cholesterol Fed Chicks[a] (Ch-SMC)

	HMG-CoA reductase activity ($pmol \cdot min^{-1} \cdot mg\ prot^{-1}$)	
Time (h)	SMC-C	SMC-Ch
2	9766.2 ± 689	6310.7 ± 520
6	7669.9 ± 888	5856.9 ± 528
12	8433.9 ± 1015	6943.4 ± 616

[a]At time zero, monolayers of three passage cultures that presented 60% confluence were washed with PBS and then fed fresh media. Cells were harvested and the reductase activity was measured at 2, 6, and 12 h after the media change. Results are mean ± SEM for 2 experiments with at least 5 values each for each experiment.

medium change not only in SMC-C but also in SMC-Ch cultures.

In senescent cultures (15 passages; Fig. 4), both SMC-C and SMC-Ch present lower reductase activity than in young cultures, and an activity peak only at 30 h after a change in medium, followed by return to basal values at 48 h. In experiments performed with LPDS, HMG-CoA reductase activity was induced 30 h after the medium change in SMC-C ($P \leq 0.05$) and 40 h after the medium change in SMC-Ch cultures ($P \leq 0.0001$) (Fig. 5).

HMG-CoA reductase gene expression in SMC-C and SMC-Ch cultures after medium change. To measure the HMG-CoA reductase gene expression in chick aorta SMC at several times after re-feeding cultures, the HMG-CoA mRNA of SMC-Ch and SMC-C of three passage cultures was measured by RT-PCR. The quantification of HMG-CoA reductase mRNA showed that the mRNA levels from young SMC-C cultures rose markedly after 16 h of medium change ($P \leq 0.0001$), returning to basal values by 56 h (Fig. 6). This rise in the number of HMG-CoA reductase mRNA molecules occurred also in young SMC-Ch cultures after a change of medium but registered far lower levels with smaller increases in reductase mRNA after 8 h of medium change in SMC-Ch ($P \leq 0.001$) (Fig. 6).

Under normal culture conditions, HMG-CoA reductase activity varied widely among experiments. To examine whether the cyclic fluctuation found in HMG-CoA reductase activity after medium change in SMC-C cultures occurred also in SMC-Ch, and to determine their possible correspondence with changes in mRNA concentration, we prepared three passage cultures isolated from cholesterol-fed chicks (SMC-Ch) and control-fed chicks (SMC-C). The reductase activity was measured after medium change (time zero) in SMC-C and SMC-Ch cultures at different times during 40 h. A parallel experiment was performed to measure the reductase mRNA concentration (Fig. 7). Although the magnitude of the fluctuations varied somewhat among SMC-C and SMC-Ch (Fig. 7A), increases of reductase activity fluctuated over the study period. The greatest change and the highest reductase activity occurred in SMC-C with cultures of three passages vs. SMC-Ch (Fig. 7A). Figure 7B shows a comparison in HMG-CoA reductase mRNA in both SMC-C and SMC-Ch cultures; the increases of reductase mRNA after medium change in SMC-Ch and SMC-C young cultures was similar to the results shown in Figure 6. SMC-C cultures showed a large rise after a change in medium, returning to basal values afterward. However, there was varia-

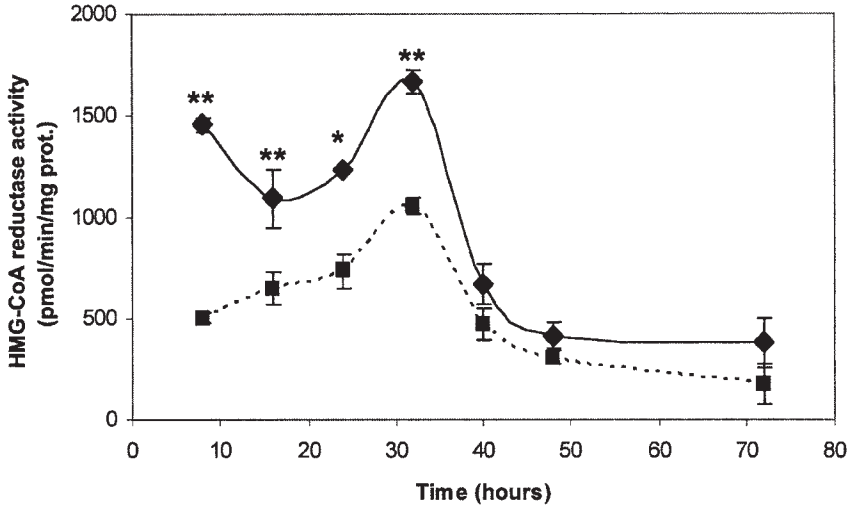

FIG. 4. Variations in HMG-CoA reductase activity of 15 passages SMC-C (◆) and smooth muscle cell–cholesterol-fed (SMC-Ch) (■) unsynchronized cultures after media change supplemented with FBS. At time-zero, cultures with 50–60% of confluence were washed with PBS and then fed with fresh media. Cells were harvested at each time point, and the HMG-CoA reductase activity was determined. Results are mean ± SEM for 3 experiments. *$P \leq 0.001$, **$P \leq 0.0001$ vs. SMC-Ch. For other abbreviation see Figure 1.

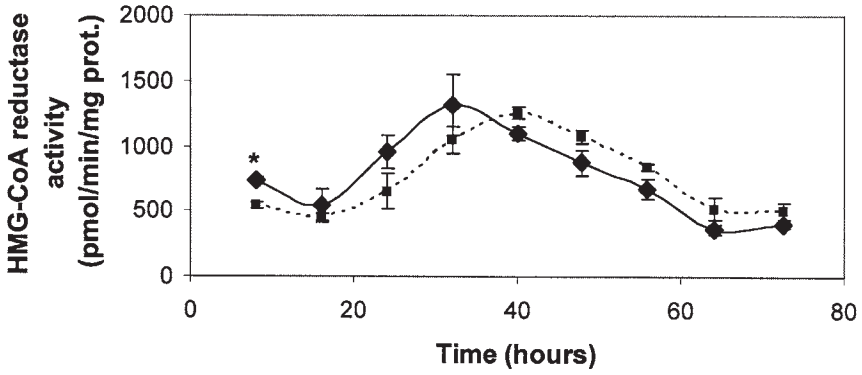

FIG. 5. Effect of media change supplemented with LPDS on HMG-CoA reductase activity of three passages SMC-C (◆) and SMC-Ch (■) unsynchronized cultures. At time zero, cultures with 50–60% confluence were washed with PBS and then fed fresh media with LPDS. Cells were harvested at each time point, and the HMG-CoA reductase activity was determined per mg protein (prot.). Results are mean ± SEM for three experiments. *$P \leq 0.001$ vs. values in SMC-Ch cells at the same time point. For abbreviations see Figures 1, 3, and 4.

tion in the time of rise of these mRNA concentrations with regard to Figure 6. Moreover, SMC-Ch presents the smallest changes and the lowest number of mRNA molecules (Fig. 6).

DISCUSSION

The primary purpose of the present study was to verify and extend the evidence for the dual role of reductase activity fluctuations related to the cell cycle and to food intake in the SMC-C/SMC-Ch cultures. We also wanted to determine specifically whether the alteration of steady-state levels of mRNA for HMG-CoA reductase could also account for cyclic fluctuations in cell cultures, as has been shown for diurnal variations in rat liver (6), or whether this cyclic fluctuation must be predominantly controlled by posttranscriptional regulation of HMG-CoA reductase activity, as it has been claimed previously (47).

The major finding of the present study is that HMG-CoA reductase activity in synchronized and unsynchronized cell cultures underwent marked fluctuations over a long period (72 h) after the change in medium. These fluctuations are not associated with feeding the cells as reported previously (14), since cultured cells were fed only at time zero. These results partially agree with the previous observation in synchronized cell cultures, with two peaks occurring 24 h prior to or at the time of the S phase (24), and also agree with the cyclic variations of reductase activity in dThd block synchronized cells with maximal activity in S or G_2/M phases (26). However, these findings contrast with those of Tavangar *et al*. (14), who stated that this variation in reductase activity is associated only with feeding the cells.

Since the culture does not suffer any re-passage, the growth of the cells is markedly different throughout the experiment.

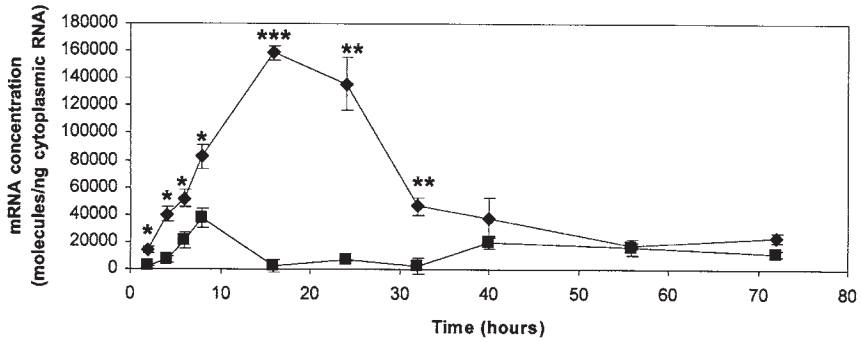

FIG. 6. Comparison of the effect of media change on HMG-CoA reductase mRNA in SMC-C (◆) and SMC-Ch (■) cultures. At time zero, three passage cultures that presented 50–60% confluence were washed with PBS and then fed fresh media. Cells were harvested at each time point, and the HMG-CoA reductase mRNA levels were quantifed by competitive reverse transcription polymerase chain reaction. Results are mean ± SEM for 3 experiments. *$P \leq 0.05$, **$P \leq 0.001$, ***$P \leq 0.0001$ vs. values in SMC-Ch cells at the same time point. For abbreviations see Figures 1 and 4.

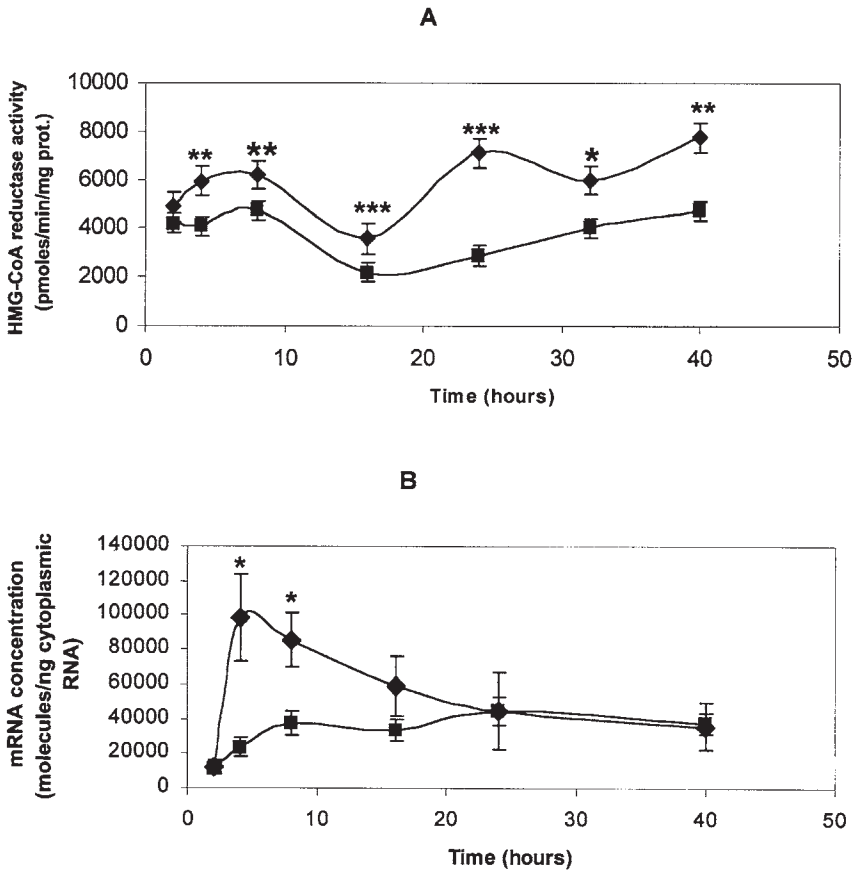

FIG. 7. Comparison of the effect of media change supplemented with FBS on HMG-CoA reductase (A) activity, and (B) mRNA in SMC-C (◆) and SMC-Ch (■) unsynchronized cultures. At time-zero, 3 passage cultures with 50–60% confluence were washed with PBS and then fed fresh media. Cells were harvested at each time point, and the HMG-CoA reductase activities and the HMG-CoA reductase mRNA levels were quantified. Results are mean ± SEM for 3 experiments. (A) $*P \leq 0.01$, $**P \leq 0.001$, $***P \leq 0.0001$; (B) $*P \leq 0.05$ vs. SMC-Ch. For abbreviations see Figures 1 and 4.

Initially the cultures were not confluent and the cells were in the logarithmic growth phase. At the end of the experiment, when confluence was reached, they were in the stationary growth phase. Peaks of reductase activity between 55 and 72 h after the change of medium, when the cultures reach confluence, could contrast with previous results of a decrease in reductase when cultures reach confluence (28,29) or a maximal activity in S or G_2/M phase. However, in SMC cultures the proportion of the cells in S phase was the same between cultures with 50–60% confluence and 100% confluence. In late-passage cultures (senescence), the HMG-CoA reductase activity was down-regulated and fluctuations were found, with activity peaking at 16 h after the medium change and returning to basal values afterward, as described previously (14). In addition, senescent cell cultures underwent apoptosis since they presented nuclear fragmentation (DNA leader) (results not shown). It could be speculated that the inhibition of HMG-CoA reductase in senescent cultures has the same effect of inhibitors of reductase (statins) that induce apoptosis in vascular SMC (48). In this regard, there is evidence of apoptosis in SMC from human atherosclerosis and in an experimental model (49,50). Thus, senescent cells could vary in the probable molecular mechanism that leads to the fluctuations in the reductase activity, with differences in behavior between senescent and young cells resulting from the metabolic change that cells in culture suffer with time. These alterations have been interpreted as an accelerated process of senescence due to the "proliferate stress" of the cells in culture (51), similar to the natural senescence of the cells within the animal. One important exception is transformed cells, such as the established cell line, which does not undergo senescence (36). Previous studies with confluent cultures of the established cell line (14) described only a peak after medium change; however, they measured reductase activity only during the first 24 h in confluent cultures.

The concentration of the reductase mRNA in SMC cultures showed a marked increase only after 16 h of exposure to fresh media in unsynchronized cell cultures. To confirm that cyclic fluctuations of the reductase activity are not correlated with

fluctuations of mRNA concentrations, these experiments were repeated by assaying reductase activity and concomitantly measuring the mRNA concentration. Although with wide variations in amplitude and periods of the cyclic activity among experiments, similar results were found in three additional experiments (not shown). We confirmed our observation of several peaks for reductase activity and one peak for mRNA concentration. The first peak of reductase activity could be explained by correlating feeding cells with the mRNA concentration rise, but the following fluctuations in reductase activity, which did not correlate with fluctuations in mRNA concentration, could not be explained by a contribution of exogenous nutrients and were strongly suggestive of a posttranscriptional modulation of the HMG-CoA reductase activity. The posttranscriptional modulation could be related to DNA synthesis, because the S phase of the cell cycle in confluent cultures was the same as in 50% confluence. The posttranscriptional modulation of reductase activity has previously been suggested (52) for feedback regulation of hepatic HMG-CoA reductase activity by dietary cholesterol.

Our results from the experiments with SMC-C vs. SMC-Ch also showed the effect of a high-cholesterol diet on the cyclic fluctuations of HMG-CoA reductase. In fact, we showed previously that SMC-Ch develops the characteristics of macrophage-like foam cells after 30 d in culture under the same culture conditions as the SMC-C (30). The SMC cultures used in these studies were young (three passages), and the SMC-C and SMC-Ch characteristics studied previously were identical even with respect to the intracellular cholesterol concentration that would become double in SMC-Ch after 20 d in culture, but it was identical during the first 14 d of culture (30). In the present study, we found a sharp decrease in HMG-CoA reductase activity and mRNA concentration in SMC-Ch vs. SMC-C in three-passages cultures, this not being dependent on the intracellular cholesterol concentration. These observations were repeated in experiments with senescent cultures except that, in the experiments with LPDS, the induction of reductase activity was similar in the two cultures.

These results suggest that the cholesterol diet induces change in HMG-CoA reductase gene expression in SMC cultures before the morphologic transformation of the SMC in the artery wall (53). SMC *in vivo* were shown to increase cholesterol biosynthesis significantly and decrease the intracellular cholesterol efflux associated with phenotype changes in SMC in culture (54,55). In this work, however, it should be noted that we are making a comparison with an SMC-C culture that underwent the same phenotype modulation by cultured conditions as the SMC-Ch. Thus, changes in HMG-CoA reductase activity and gene expression at transcriptional levels in SMC-Ch must be due to the cholesterol feeding of the chicks. These observations may be explained by the nutritional control by sterol regulatory element-binding proteins (SREBP), a family of transcription factors that bind to the sterol regulatory element (SRE) in the promoter of the HMG-CoA reductase gene. These transcription factors have been defined as key regulators of nutritional homeostasis and, in response to diet, SREBP gene regulation depends on changes in the levels of oxysterols, insulin/glucose, and PUFA (56).

Although the reductase activity and the mRNA concentration proved lower in SMC-Ch than in SMC-C, the fluctuations in the activity and the increase in reductase mRNA concentration after the medium change do not disappear in SMC-Ch. Even in senescent cultures, which present a high intracellular concentration of cholesterol in SMC-Ch (30), the reductase activity was not completely inhibited. Moreover, in cultures with LPDS induction the reductase activity underwent the same rise in SMC-C as in SMC-Ch after the change of the medium with FBS. These data agree with our results concerning the synthesis of cholesterol in the previously reported model of SMC-C/SMC-Ch cultures (31) and contrast with the general established decrease of HMG-CoA reductase activity and mRNA in hypercholesterolemic animals (57,58) or the depression of the diurnal rhythm after cholesterol-diet feeding (12–16). A possible explanation is that SRE function as conditional positive elements and are necessary for activated transcription in sterol-deprived cells but are not required for basal transcription that occurs in cholesterol-loaded cells (55). These differences of the reductase activity and the mRNA concentration between SMC-C and SMC-Ch are more important in young cultures than in senescent ones. Since in three-passage cultures the intracellular cholesterol in SMC-C and SMC-Ch was the same, other explanations must be considered regarding the transformation of the aortic SMC by cholesterol diet in the chick prior to cell harvest.

The failure to abolish the fluctuations of reductase activity in young SMC-C cultures could have a plausible explanation in the continued cell proliferation after confluence (30). The cell cycle of SMC-C in different confluence states of the cultures presents the same proportion of the cells in the S phase as when 100% confluence of the culture is reached. The mRNA concentration from young SMC-C cultures presents a peak only several hours after the medium change. However, it decreases to baseline when the cultures are confluent, so at transcriptional levels the HMG-CoA reductase regulation is affected by the degree of confluence of the culture and could be unrelated to S phase of the cell cycle.

In conclusion, in cultured aortic SMC, the HMG-CoA reductase activity shows fluctuations not associated with feeding the cells and that must be modulated at the posttranscriptional level. This fluctuation in activity changes with the culture conditions: young vs. senescent; synchronized vs. unsynchronized; LPDS vs. FBS; and confluent vs. nonconfluent. Moreover, a high-cholesterol diet in the animals before obtaining the SMC cultures induced changes in the HMG-CoA reductase gene expression. Thus, the mRNA concentration was down-regulated in SMC-Ch vs. SMC-C. Since the nutritional culture conditions, the intracellular cholesterol concentration, and the ultrastructural morphology were the same during the first days of culture, this change in reductase gene expression must have been induced by the cholesterol diet in the aortic SMC *in vivo*, probably at the SREBP gene-expression level. New studies would be necessary to demonstrate the implication of the SREBP's gene expression as well as if new protein synthesis is

required for the increase in the enzyme activity or if some peaks of activity correlate with other regulatory events. Changes in mRNA stability may also contribute to the changes observed in HMG-CoA reductase mRNA.

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Polyunsaturated Fatty Acids Regulate Cytokine and Prostaglandin E_2 Production by Respiratory Cells in Response to Mast Cell Mediators

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ABSTRACT: A protective association between breastfeeding and the development of bronchial asthma has been demonstrated. However, a mechanism remains unclear. FA present in human milk but rare in infant formula have been associated with marked immunological modulation as well as some indications of protection from asthma development. We examined the effect of *in vitro* manipulation of membrane phospholipid on the production of cytokines and prostaglandin (PG)E_2 by respiratory epithelial cells (A549) in response to stimulation by mast cell mediators of allergic disease [histamine, tumor necrosis factor (TNF)-α, interleukin (IL)-4 and IL-5]. DHA and CLA significantly decreased the production of IL-8 in response to stimulation by TNF-α [2907 ± 970 (DHA) and 6471 ± 1203 (CLA) vs. 12,287 ± 2309 (control) pg/mL; $P \leq 0.05$, mean ± SEM], whereas both EPA and DHA reduced histamine-stimulated RANTES (regulation on activation, T cell-expressed and -secreted) production [2314 ± 861 (EPA) and 877 ± 326 (DHA) vs. 8526 ± 1118 (control) pg/mL; $P \leq 0.03$]. PGE_2 released in response to histamine was decreased by n-3 [1305 ± 399 (α-linolenic acid), 406 ± 73 (EPA), and 265 ± 32 (DHA) vs. 9324 ± 3672 (control) pg/mL; $P \leq 0.05$] and increased by n-6 [18,843 ± 4439 (arachidonic acid) vs. 9324 ± 3672 (control) pg/mL; $P = 0.02$], with CLA producing a decrease of the same magnitude as DHA [553 ± 126 (CLA) vs. 9324 ± 3672 (control) pg/mL; $P = 0.03$]. This study demonstrates the potential for immunological manipulation of the respiratory epithelium by FA *in situ* during allergic responses and suggests that further investigation into FA intervention in infants *via* human milk or supplemented infant formula, to prevent the development of allergic disease, may be worthwhile.

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Systematic reviews and meta-analyses of prospective studies examining the association between breastfeeding and the development of bronchial asthma found a lower incidence with exclusive breastfeeding during the first months of life (1,2). However, the mechanism by which breastfeeding may confer this benefit to the infant is unclear. Fat, at 4%, is second only to lactose among the nonaqueous constituents of human milk (3). Many of the PUFA present in human milk but absent from some infant formulas alter the production of cytokines and eicosanoids in peripheral blood in models of disease and inflammation, as well as demonstrate beneficial effects in diseases such as cancer, atherosclerosis, and rheumatoid arthritis (4). There have also been some indications of positive effects on asthma (5). These studies have examined asthma incidence in relation to dietary habits and found that diets high in the n-3 PUFA eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which are derived predominantly from fish, are associated with a lower prevalence of asthmatic disease.

Human milk has, until recently, been an infant's only source of preformed EPA and DHA. The amounts received by each breastfed infant are variable due largely to long-term maternal diet. However, other factors such as maternal atopy may also contribute to significant differences in the composition of PUFA in human milk (6,7). These differences may contribute to an explanation for discrepancy in studies examining the protective effects of breastfeeding on the development of allergic disease.

Allergic disease of the airways is driven by cytokines produced in response to allergen presentation by macrophages and dendritic cells to T lymphocytes. The predominant T lymphocyte present, T helper (Th)1 or Th2, determines the course that the processing of allergens will take (8,9). The source of the cytokines that drive the development of the Th2 lineage responsible for the development of allergic disease includes both epithelial cells and respiratory tissue mast cells. The production of inflammatory mediators such as prostaglandin (PG)E_2 by the airway epithelium and surrounding tissue leukocytes results in a pronounced cellular infiltrate of the airway epithelium and submucosa by lymphocytes, eosinophils, and mast cells (10). On activation in the airways, these cells produce additional mediators that can further exacerbate inflammation and enhance development of the Th2-driven response. Mast cells are one of the primary contributors to this *via* release of pre-formed histamine, tryptase, and chymase, as well as by producing cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-4, and IL-5. These mast cell products are responsible for increasing vascular permeability, Th2 lymphocyte development, and

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Abbreviations: A549, human transformed alveolar epithelial cell line; AA, arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; EIA, enzyme immunoassay; IL, interleukin; LA, linoleic acid; LCPUFA, long-chain PUFA; OA, oleic acid; PGE_2, prostaglandin E_2, RANTES, regulation on activation, T cell-expressed and -secreted; Th, T helper; TNF, tumor necrosis factor.

eosinophil attraction leading to bronchoconstriction; and the up-regulation of a number of secondary inflammatory and chemotactic products including PGE_2, IL-1β, TNF-α, IL-8, and regulation on activation, normal T cell-expressed and -secreted (RANTES).

This study used a model developed in our laboratory (11,12) of *in vitro* FA manipulation in a respiratory cell line (A549) to examine the hypothesis that changes in membrane PUFA would significantly alter the production of inflammatory mediators in response to mediators of allergic disease. We examined the effect of manipulation of membrane phospholipid on the production of cytokines (IL-1β, TNF-α, IL-8, RANTES) and PGE_2 by respiratory epithelial cells in response to stimulation by mast cell mediators of allergic disease (histamine, TNF-α, IL-4, IL-5).

EXPERIMENTAL PROCEDURES

Mast cell mediator stimulation of A549 Cells. Human transformed alveolar respiratory epithelial cells (A549) at 2.5×10^5 cells/well were cultured in 24-well cell culture plates (Greiner Labortechnik GmbH, Frickenhowsen, Germany) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% heat-inactivated FBS (JRH Biosciences, Lenexa, KS). At confluence (24 h), supernatants were removed and cells cultured for a further 24 h with or without the human recombinant cytokines IL-5 (20 ng/mL; Peprotech, Rocky Hill, NJ), IL-4 (20 ng/mL), or TNF-α (2 ng/mL; R&D Systems Inc., Minneapolis, MN) or 16 mM histamine (Sigma Chemical Company, St. Louis, MO) in serum-free DMEM in triplicate. At 24 h, each culture plate was subjected to a single freeze-thaw cycle to lyse the cells. Culture supernatants were then harvested, spun at ~2200 × *g* to pellet cell debris, and aliquoted before storing at –80°C until assay for cytokine or PG production.

Mast cell mediator stimulation of long-chain PUFA (LCPUFA)-supplemented cells. A549 cells were cultured as above in the presence or absence of 10 or 50 μg/mL α-linolenic acid (ALA; 18:3n-3), linoleic acid (LA; 18:2n-6), EPA, arachidonic acid (AA; 20:4n-6), DHA, CLA (Fig. 1) (Nu-Chek-Prep Inc., Elysian, MN), or oleic acid (OA; 18:1n-9) (ICN Pharmaceuticals Inc., Costa Mesa, CA) as FFA solubilized in 100% ethanol. These FA concentrations result in approximately 50% incorporation and maximal incorporation, as described previously (11,12). Briefly, incorporation of FFA was determined in separate triplicate experiments. Cellular lipids were extracted using the method described by Broekhuyse (13). The phospholipid fraction was separated *via* TLC, lipids were transesterified, and FAME were separated and quantified as described previously (11) using a Hewlett-Packard 6890 gas chromatograph fitted with a 50 m capillary column (0.33 mm i.d.) coated with BPX-70 (0.25 mm film thickness; SGE Pty Ltd., Victoria, Australia). The injector temperature was set at 250°C and the FID temperature at 300°C. The initial oven temperature was 140°C and was programmed to rise to 220°C at 5°C/min. Helium was used as the carrier gas at a velocity of 35 cm/s. FAME were identified based on the retention time of authentic lipid standards (Nu-Chek-Prep Inc.). At the commencement of this project, CLA was only available as a combined mixture of approximately five isomers (14). The use of this blend of CLA isomers, while preventing analysis of individual effects, most closely mimics the CLA component of human milk, which contains measurable quantities of all but the 11-*cis*,13-*trans* 18:2 CLA isomer, with 9-*cis*,11-*trans* 18:2 CLA constituting the largest fraction (15). The relative incorporation of each of these isomers is described by Figure 1. Correct identification of each isomer was confirmed using a Hewlett-Packard 6890 GC-Mass Spectrometer. At confluence cells were cultured with or without TNF-α or histamine (as above), with or without the appropriate FFA (10 and 50 μg/mL). At 24 h, cells were lysed and supernatants collected and stored as above.

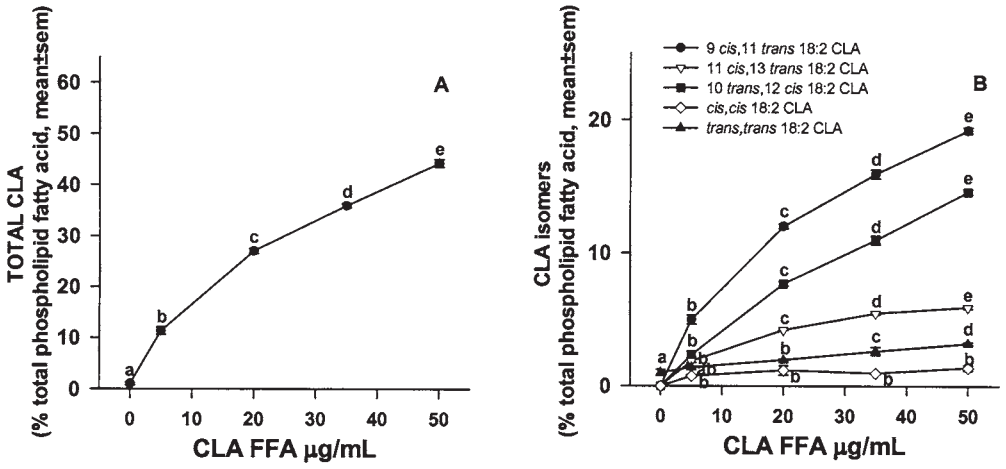

FIG 1. Cell membrane phospholipid CLA from respiratory epithelial cells incubated with a range of concentrations of CLA as FFA. Results from 3 or 4 experiments are expressed as a relative percentage of the total FA (mean ± SEM). Points with different superscripts indicate significant differences ($P < 0.05$). (A) Total incorporated CLA. (B) Individual amounts of each CLA isomer.

PG assay. Cell culture supernatants were assayed for PGE_2 by competitive enzyme immunoassay (EIA) (Prostaglandin E_2 EIA Kit—Monoclonal; Cayman Chemical Company, Ann Arbor, MI). The limit of detection for the EIA was 8 pg/mL. Samples were diluted at least 1:10 in the EIA buffer supplied as per the manufacturer's instructions.

Cytokine assays. Cell culture supernatants were assayed for IL-1β, TNF-α (Endogen, Rockford, IL), IL-8 (BD Pharmingen, San Diego, CA) and RANTES (R&D Systems) using double antibody sandwich ELISA as described previously (12). The limit of detection for each ELISA was 8, 10, 20, and 4 pg/mL, respectively. Samples were diluted 1:10 and 1:20 for the measurement of IL-8 and RANTES, respectively.

Statistical analysis. Data are presented as mean ± SEM. Differences between FA and mediator treatments were examined using Independent-Samples *T*-test or one-way ANOVA followed by LSD (least significant difference) *post-hoc* analysis. All analyses were performed using SPSS for Windows 10.0 (SPSS Inc., Chicago, IL).

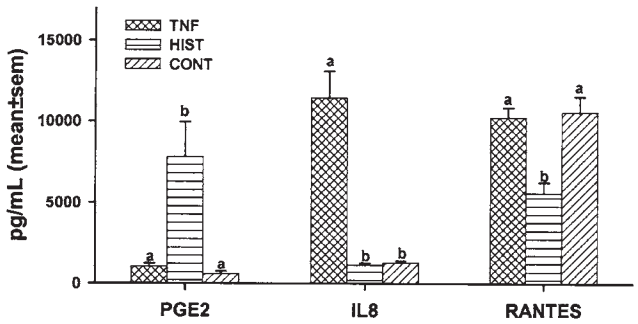

FIG. 2. Effect of incubation with mast cell mediators [tumor necrosis factor-α (TNF-α), 2 ng/mL; histamine (HIST), 16 mM; no stimulant control (CONT)] on prostaglandin $(PG)E_2$, interleukin (IL)-8, and RANTES (regulation on activation, T cell-expressed and -secreted) production by A549 cells after 24 h of culture. Results from 6 experiments are expressed as pg/mL (mean ± SEM). Points with different superscripts indicate significant differences ($P < 0.05$).

RESULTS

Production of cytokines and PGE_2 in response to stimulation with mast cell mediators. Incubation with TNF-α significantly increased the production of IL-8 by A549 cells above that of control, or no stimulant, levels (Fig. 2). Similarly, histamine significantly increased PGE_2 production while also eliciting a decrease in RANTES production when compared with control levels. There was no effect on the production of IL-8 by A549 cells in response to incubation with histamine. Similarly, there was no effect of TNF-α on the production of PGE_2 or RANTES. Stimulation of the cells with IL-4 and IL-5 did not elicit a significant increase in production of any of the mediators tested (data not shown). Although there was constitutive production of the inflammatory cytokine IL-1β (228 ± 79 pg/mL, mean ± SEM), there was little to no spontaneous production of TNF-α, thus resulting in a mean below the limit of detection for this assay (10 pg/mL). No difference was found in the production of either IL-1β or TNF-α between the control and those cells treated with cytokine or histamine (data not shown).

Effect of FA incorporation into the A549 cell membrane on constitutive PGE_2, IL-8, and RANTES production. The degree to which each of the FFA, excluding CLA, was incorporated into the A549 cells has been reported previously (11,12). As with all other FFA used in this project, each CLA isomer was readily incorporated dose-dependently into the cell membranes of the A549 respiratory epithelial cells. However, the incorporation of CLA continued to increase up to, and including, 50 μg/mL supplemental total CLA FFA. Each of the CLA isomers was incorporated proportionally to the content of the FFA mixture, resulting in a majority of 9-*cis*,11-*trans* CLA (~45%) and 10-*trans*,12-*cis* CLA (~35%) with the remaining isomers making up the final 20% of the total CLA increase. The incorporation of each of the FA—ALA, LA, OA, EPA, AA, DHA, and CLA—into the membranes of A549 cells at maximum incorporation (50 μg/mL FFA; 20–25% total phospholipids) (11,12) resulted in a significant decrease in the production of IL-8 when compared with approximately 50% maximum incorporation (10 μg/mL FFA) (Fig. 3B). A similar pattern was observed in the production of RANTES by A549 cells after incorporation of FFA at each concentration. However, this trend only attained statistical significance with EPA, AA, and DHA (Fig. 3C). In contrast, no significant difference in PGE_2 production between differing FFA concentrations (10 or 50 μg/mL) was found for each of the FA tested (Fig. 3A). Changes in cell membrane phospholipid resulted in significant differences in the production of each of the products examined. However, this effect was only attained at the higher FFA concentration of 50 μg/mL for the chemokines, IL-8, and RANTES, whereas the effects on PGE_2 were similar for both concentrations. Incorporation of AA resulted in a significant increase in PGE_2 production, which was also apparent at 50 μg/mL supplementation with its precursor FA LA, when compared with control (no FFA). The amount of PGE_2 produced by cells after incorporation of OA, ALA, EPA, DHA, and CLA was significantly lower than that of the cells incubated with AA at both concentrations but reached statistical significance compared with LA only at 50 μg/mL (Fig. 3A). There was no difference in the production of the chemokines IL-8 and RANTES in response to incorporation of FFA at 10 μg/mL. At 50 μg/mL supplemental FFA, the amount of both chemokines produced by cells having incorporated each of the n-3 PUFA ALA, EPA, and DHA, as well as CLA, was decreased from that of the control cells, in serum-free media. However, rather than resulting in an increased production as with PGE_2, the incorporation of the n-6 PUFA AA also manifested as a decrease in IL-8 and RANTES (Figs. 3B, 3C). The production of RANTES was similarly reduced in response to DHA incorporation when compared with production by cells incubated with all FFA treatments, with the exception of EPA (Fig. 3C).

Effect of FA incorporation into the A549 cell membrane on IL-8 production in response to stimulation with TNF-α. The n-

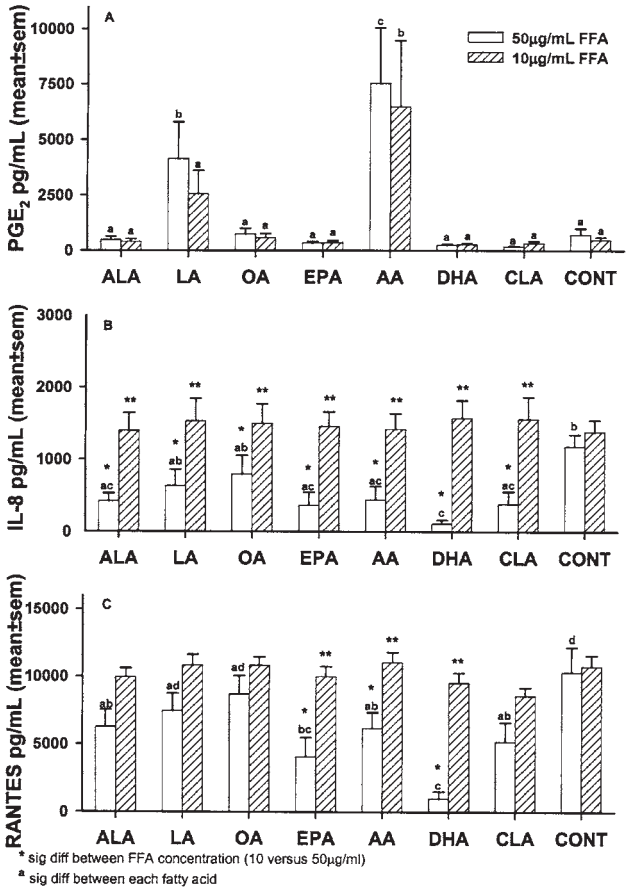

FIG. 3. Effect of incubation with PUFA [α-linolenic acid (ALA), linoleic acid (LA), oleic acid (OA), EPA, arachidonic acid (AA), DHA, CLA, no FFA (CONT)] at 10 µg/mL vs. 50 µg/mL on (A) PGE_2, (B) IL-8, and (C) RANTES production by nonstimulated A549 cells (human transformed alveolar epithelial cell line) after 24 h of culture. Control cells were incubated in serum-free medium containing equivalent ethanol to the 10 or 50 µg/mL FFA solutions, respectively. Results from 6 experiments are expressed as pg/mL (mean ± SEM). Points with different superscripts indicate significant differences ($P < 0.05$) between each FA. Asterisks (*) indicate significant differences between FFA concentrations (10 vs. 50 µg/mL). For other abbreviations see Figure 2.

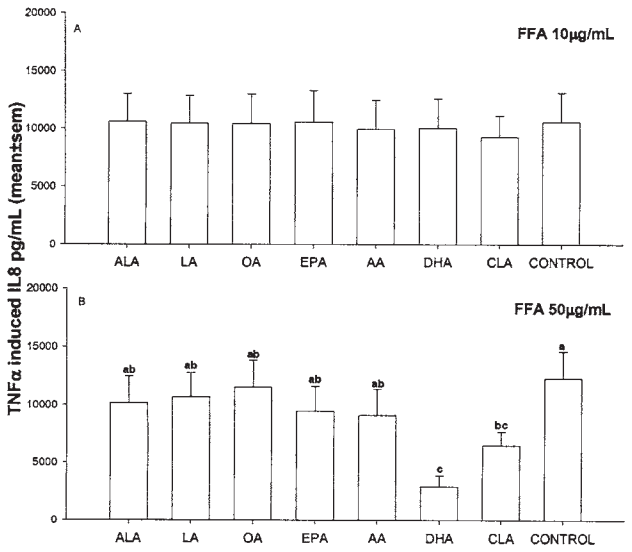

FIG. 4. Effect of incubation with PUFA (ALA, LA, OA, EPA, AA, DHA, CLA) at 10 and 50 µg/mL on IL-8 production by A549 cells after 24 h of culture with TNF-α (2 ng/mL). Control cells were incubated in the presence of TNF-α in serum-free medium containing equivalent ethanol to the 10 or 50 µg/mL FFA solutions, respectively. Results from 6 experiments are expressed as pg/mL (mean ± SEM). Points with different superscripts indicate significant differences ($P < 0.05$). For abbreviations see Figures 2 and 3.

3 FA, CLA, and AA were able to decrease the production of IL-8 by epithelial cells significantly. However, DHA and CLA only maintained this effect upon stimulation with TNF-α. At 50 µg/mL FFA supplementation, a significant difference in the TNF-α–stimulated production of IL-8 was observed between cells that had incorporated either DHA or CLA when compared with control cells incubated in the absence of FFA (Fig. 4B). No changes in TNF-α stimulated IL-8 production in response to FA incorporation at 10 µg/mL were apparent (Fig. 4A).

Effect of FA incorporation into the A549 cell membrane on PGE_2 and RANTES production in response to stimulation with histamine. PGE_2 production by cells that had incorporated exogenous FA and then were incubated with histamine demonstrated a distinct n-3-induced pattern at maximal FFA incorporation (50 µg/mL) that was only partially retained at half maximum incorporation (10 µg/mL) (Figs. 5A, 5B). At 50 µg/mL, incorporation of ALA was sufficient to reduce the production of PGE_2 significantly in response to histamine with further decreases with both EPA and DHA incorporation. Incorporation of CLA again mimicked the n-3 PUFA. In contrast, incorporation of the n-6 PUFA AA resulted in a significant increase in PGE_2 production at both 10 and 50 µg/mL supplemental FFA. Whereas PGE_2 production by LA-supplemented cells also increased when compared with all n-3 PUFA and CLA at 50 µg/mL, and DHA and CLA at 10 µg/mL, incorporation of LA did not significantly increase PGE_2 at either FFA concentration in comparison with control cells in the absence of FFA. The n-3 LCPUFA, CLA, and AA were all able to suppress the production of RANTES by A549 cells (Fig. 3C). However, EPA and DHA only maintained this effect when a mast cell mediator (histamine) was present (Fig. 5B). The effect of FA incorporation on histamine inhibition of RANTES production was only apparent at maximal FFA incorporation. Again there was an effect of n-3 LCPUFA composition with increasing EPA and DHA resulting in a decrease in RANTES production when compared with no FFA control cells. There was no effect of incorporation of any of the remaining FFA on the production of RANTES in response to incubation with histamine.

DISCUSSION

In this study, the incorporation of both n-3 and n-6 PUFA and CLA affected the production of PGE_2, IL-8, and RANTES by respiratory epithelial cells in response to exposure to products of stimulated mast cells, TNF-α and histamine.

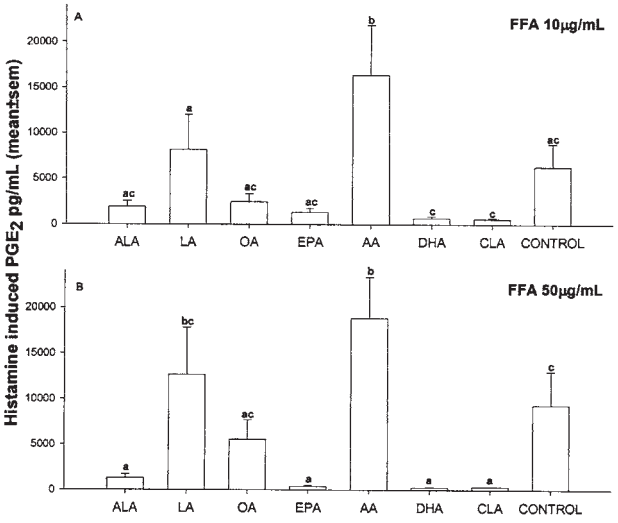

FIG. 5. Effect of incubation with PUFA (ALA, LA, OA, EPA, AA, DHA, CLA) at 10 and 50 μg/mL on PGE_2 production by A549 cells after 24 h of culture with histamine (16 mM). Control cells were incubated in the presence of histamine in serum-free medium containing equivalent ethanol to the 10 or 50 μg/mL FFA solutions, respectively. Results from 6 experiments are expressed as pg/mL (mean ± SEM). Points with different superscripts indicate significant differences ($P < 0.05$). For abbreviations see Figures 2 and 3.

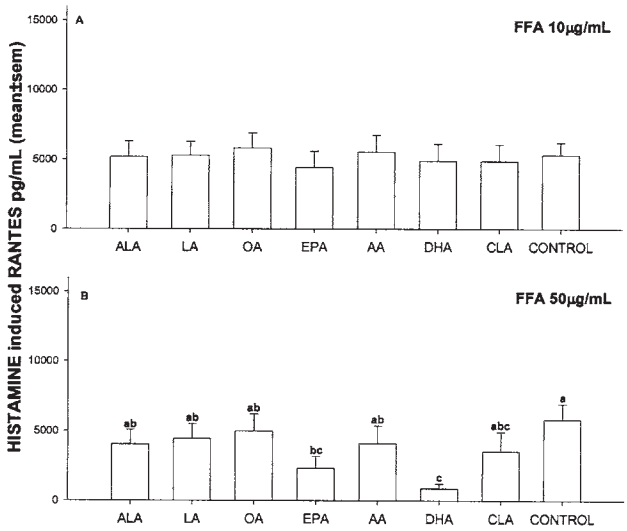

FIG. 6. Effect of incubation with PUFA (ALA, LA, OA, EPA, AA, DHA, CLA) at 10 and 50 μg/mL on RANTES production by A549 cells after 24 h of culture with histamine (16 mM). Control cells were incubated in the presence of histamine in serum-free medium containing equivalent ethanol to the 10 or 50 μg/mL FFA solutions, respectively. Results from 6 experiments are expressed as pg/mL (mean ± SEM). Points with different superscripts indicate significant differences ($P < 0.05$). For abbreviations see Figures 2 and 3.

Prostanoids such as PGE_2 play a pivotal role in the regulation of allergic airway disease through both pro- and anti-inflammatory actions. Although PGE_2 concentrations in tissues are generally low, they are rapidly increased in acute inflammation prior to the influx of leukocytes, which act to enhance prostanoid production further, usually *via* an up-regulation of cyclo-oxygenase (COX)-2 expression (16). During airway inflammation, PGE_2 acts *via* the prostanoid receptor, EP2 to stimulate smooth muscle cells resulting in vasodilation, and the EP3 receptor to induce mast cell degranulation, increasing vascular permeability. However, after the initial phase of inflammation, increasing PGE_2 levels produced by epithelium, as well as infiltrating macrophages, act to inhibit leukocyte activation and promote bronchodilation *via* the EP2 and EP4 receptors (17). PGE_2 regulation by decreasing n-6 to n-3 ratios, or by supplementation with CLA, may dampen the initial pro-inflammatory response in the allergic airway, thereby controlling the establishment of inflammation before the anti-inflammatory properties of increased PGE_2 levels come into play. Decreasing first phase PGE_2 concentrations would reduce vasodilation and vascular permeability, as well as mast cell degranulation. Alternatively or perhaps concurrently, changes in the relative amounts of eicosanoid products formed owing to the concentrations of precursor FA would result in substantial effects on the production of many cytokines. Lowering of the PGE_2 concentration owing to changes in the ratio of AA to EPA would result in decreases in the production of proinflammatory cytokines such as IL-1βb and TNF-α, as well as the Th1 cytokines IL-2 and IFN-γ (4). As IL-1β, TNF-α, and IFN-γ are all stimulants of epithelial production of chemokines (18,19), this decrease in production of stimulant cytokines would thereby reduce production of IL-8 and RANTES.

Chemotactic factors, such as IL-8 and RANTES, produced by the respiratory airways in response to degranulation of tissue mast cells during allergic airway disease, also play an important role in the progress of airway inflammation. Release of mast cell products in response to binding of either allergen-specific or, prior to sensitization, nonspecific IgE to the $\underline{F}_{C\varepsilon}RI$ (high-affinity IgE receptor), contributes not only to the development of chronic inflammation but also to the initiation of allergic sensitivity (20). By promoting the influx of large numbers of activated leukocytes into the airways, these chemotactic factors contribute to the increased concentrations of inflammatory eicosanoids and cytokines and to the development of structural airway changes. Increases in epithelial IL-8 production due to the presence of mast cell-derived TNF-α would result in elevated granulocyte (neutrophil, basophil, and eosinophil) numbers migrating to allergic airways. There, both TNF-α and IL-8 would activate the incoming granulocyte populations, resulting in the further release of chemotactic and inflammatory mediators (21). Conversely, the release of preformed granules containing histamine from airway mast cells may inhibit the production of RANTES by respiratory epithelium. RANTES is responsible for inducing the migration of mast cells, eosinophils, basophils, macrophages, and T lymphocytes into the airways, resulting in increases in PGE_2, allergic inflammation, bronchial hyperresponsiveness, and eosinophil activation, as well as additional release of histamine by mast cells and basophils (22–24). Therefore, decreases in

the production of both IL-8 and RANTES by enhanced tissue n-3 or CLA FA incorporation may substantially diminish cellular infiltration in the airways and the resultant inflammatory responses to allergens, thereby significantly reducing the progression of respiratory hyperresponsiveness to asthma.

Although FA effects on the production of PG such as PGE_2 by peripheral blood leukocytes are well documented (4), the literature contains few studies relating to FA affecting production of PG in the lung (25,26). Perhaps the most relevant studies have determined that rat pups from dams fed diets supplemented with fish oil throughout pregnancy and lactation have significantly depressed PGE_2 production in their lung tissue compared with those born to dams on control diets (27,28). These experiments confirm *in vivo* the potential application of FA supplementation on inflammatory mediator production in the lungs of infants during lactation.

A number of studies have found significantly lower EPA and DHA concentrations in the milks of nonatopic mothers whose infants develop atopy, resulting in lower plasma n-3 levels and higher n-6/n-3 ratios, indicating an n-3-mediated affect (29) and a link between the early infant diet and the development of allergic disease (30–35). However, dietary studies in children and adults have produced conflicting results, with those examining n-3 intake *via* consumption of fish demonstrating some small but positive effects on recurrent airway disease (airway hyperresponsiveness, wheeze, or asthma) (36) but with placebo-controlled supplementation studies showing mixed results especially on established asthmatic disease (37-40). These studies provide preliminary data that may encourage speculation for the requirement for FA intervention during infant development or over a longer term via human milk or supplemented infant formula, which also provides a source of CLA. High dietary intake of CLA and n-3 FA may be required during early immune maturation and first exposures to allergens in order to modulate the immune system and thereby prevent the development of allergic disease rather than to treat symptoms of established conditions.

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Enhanced Incorporation of n-3 Fatty Acids from Fish Compared with Fish Oils

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ABSTRACT: This work was undertaken to study the impact of the source of n-3 FA on their incorporation in serum, on blood lipid composition, and on cellular activation. A clinical trial comprising 71 volunteers, divided into five groups, was performed. Three groups were given 400 g smoked salmon (n = 14), cooked salmon (n = 15), or cooked cod (n = 13) per week for 8 wk. A fourth group was given 15 mL/d of cod liver oil (CLO) (n = 15), and a fifth group served as control (n = 14) without supplementation. The serum content of EPA and DHA before and after intervention revealed a higher rise in EPA and DHA in the cooked salmon group (129% rise in EPA and 45% rise in DHA) as compared with CLO (106 and 25%, respectively) despite an intake of EPA and DHA in the CLO group of 3.0 g/d compared with 1.2 g/d in the cooked salmon group. No significant changes were observed in blood lipids, fibrinogen, fibrinolysis, or lipopolysaccharide (LPS)-induced tissue factor (TF) activity, tumor necrosis factor-α (TNFα), interleukin-8 (IL-8), leukotriene B_4 (LTB_4), and thromboxane B_2 ($T\times B_2$) in whole blood. EPA and DHA were negatively correlated with LPS-induced TNFα, IL-8, LTB_4, $T\times B_2$, and TF in whole blood. In conclusion, fish consumption is more effective in increasing serum EPA and DHA than supplementing the diet with fish oil. Since the n-3 FA are predominantly in TAG in fish as well as CLO, it is suggested that the larger uptake from fish than CLO is due to differences in physiochemical structure of the lipids.

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Seafood provides an excellent source of high biological-value proteins, peptides and amino acids, unsaturated EFA, vitamins, antioxidants, minerals, and trace metals, all with various beneficial effects on important physiological factors.

In the late 1970s the groundbreaking observation of a significantly reduced prevalence of cardiovascular disease (CVD) in Greenland Eskimos living on their traditional diet compared with Eskimos living in Denmark suggested that intake of long-chain n-3 PUFA from fish and marine mammals may protect against CVD (1). The biological effects of consuming fish-derived PUFA have been intensively explored (2–6). These studies have lent support to the notion that n-3 PUFA might affect several cellular processes known to be important in the development of coronary heart disease, stroke, etc. (7,8). Hence, there is evidence that PUFA protect against CVD and other conditions where cellular inflammatory processes are involved, even though the beneficial effects of fish-derived n-3 PUFA recently have been questioned in a meta-analysis (9). Most studies have used fish oils, but the natural source rich in these PUFA is fatty fish. Unfortunately, the doses administered in most studies exceed what is typically found in the diet (10). In contrast, considerable vascular benefits from modest fish consumption have been observed (11,12). It is well accepted that Western populations consuming little seafood have a high prevalence of CVD, in contrast to people in Japan and Greenland with higher intakes of seafood. Although it has been suggested that the bioavailability of PUFA might be better from fish compared with dietary supplements (13), these issues needs further investigation.

Based on the questions raised concerning the beneficial effects of fish oil-derived FA and the interesting observation of Visioli *et al*. (13), it is important to confirm and extend their study. Therefore, a study was undertaken to compare the effects of intake of four fish-derived sources of n-3 FA—three rich sources: raw fatty fish (smoked salmon), cooked fatty fish (salmon fillet), or fish oil (cod liver oil: CLO); and one poor source: fish low in n-3 FA (cod fillet)—on the uptake of n-3 FA in blood, lipid composition in blood, and functional properties of blood cells, as measured by the potential of lipopolysaccharide (LPS) to generate activation products in whole blood.

STUDY DESIGN AND EXPERIMENTAL PROCEDURES

Study design. Seventy-one healthy volunteers gave informed consent to the study, which had been approved by the regional Ethical Committee. The test subjects were divided into five groups, which were given, respectively, 400 g of smoked salmon (farmed *Salmo salar*) per week (14 subjects), 400 g salmon fillet per week (15 subjects), 400 g cod (*Gadus morhua*) fillet per week (13 subjects), or 15 mL CLO per day (15 sub-

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Abbreviations: apoA, apolipoprotein A-1; apoB, apolipoprotein B; apoE, apolipoprotein E; CLO, cod liver oil; CVD, cardiovascular disease; EIA, enzyme immunoassay; HRP, horseradish peroxidase; IL-8, interleukin-8; LPS, lipopolysaccharide; LTB4, leukotriene B4; MPV, mean platelet volume; RBC, red blood cells; TF, tissue factor; TNFα, tumor necrosis factor α; TxB2, thromboxane B2.

jects). The raw cod and salmon fillets were prepared by the participants themselves, according to traditional Norwegian meal-preparation techniques.

Fourteen subjects were controls; they were asked not to change their diet during the study. All volunteers were told strictly to follow the same diet as prior to the study, except for the subjects in the three fish groups, who were asked to substitute regular diet items with their respective meal of fish.

Drawing of blood. Venous blood was drawn from the antecubital vein into a sterile plastic syringe (20 mL) using 19G sterile needles. Part of the blood volume was added to a plastic test tube containing heparin (10 U/mL blood) to be used for the LPS activation, some of the blood was collected in vacutainer tubes containing citrate (0.006 M Na_3citrate, 0.004 M citric acid final concentration), and 5 mL of the blood was added to a glass vacutainer containing 15% K_3EDTA (0.054 mL/4.5 mL) to be used for the cell counts. Blood was drawn after an overnight fast between 8:00 and 10:00 AM at the start of the study and at the end of the study after 8 wk intervention.

Isolation of plasma and serum. Plasma was isolated by centrifugation of blood collected in citrate at 1500 × *g* for 10 min at room temperature. The resulting top plasma layer was divided into five test tubes and frozen at –70°C until assay analysis. Serum was prepared by clotting whole blood in glass tubes for 1 h at 37°C, followed by the centrifugation at 1500 × *g* for 10 min at room temperature.

Lipids in serum. Serum was tested for total cholesterol (enzymatic colorimetric test, cholesterol esterase, and cholesterol oxidase), HDL-cholesterol (after precipitating chylomicrons, VLDL, and LDL with phosphotungstic acid and Mg^{2+}), TAG (enzymatic colorimetric test, lipoprotein lipase/oxidation/-peroxidase). The analyses were done in an Axon autoanalyzer (Technicon/Bayer). Apolipoprotein A-1 (apoA), apolipoprotein B (apoB), and apolipoprotein E (apoE) were determined in a Beckman Nephelometer Immunonephelometric method (Beckman Immage, Fullerton, CA), using reagents from Dako (Glostrup, Denmark).

FA in serum. Total FA in serum were determined after extraction by using a modified Folch method (chloroform/methanol/KCl aq., 8:4:3) and methylation (BF_3 in methanol) as described in previous studies (14,15). The FA were analyzed by capillary GLC using a Fisons Carlo Erba 8340 (Fisons Instruments, Beverly, MA) gas chromatograph with a 50 m × 0.25 mm Chrompack CP-Sil 88 CB capillary column (Varian Inc., Palo Alto, CA). The injector temperature was 240°C, and the oven was programmed as follows: 80°C (1 min); 20°C/min to 170°C (0 min); 3°C/min to 200°C (0 min); 5°C/min to 240°C (15 min). The temperature of the FID was 270°C. The signal was analyzed by Agilent's EZchrom software (Santa Clara, CA) and compared with mixtures of FA supplied by Supelco (Bellefonte, PA).

The individual FA in serum were calculated by use of 17:0 as an internal standard and expressed as mmol/L. The identification was based on retention times.

Lipids and FA in fish muscle. The fat content of fish samples was determined by AOCS method Ba 3-38 (16) using a Soxhlet extractor (Soxtec System HT6; Tecator, Höganäs, Sweden) and the FA composition of the samples was determined by using GC after direct acidic methylation of fish (17) and extraction of lipid samples using a modified Bligh and Dyer (18) method. Further analytical procedures are described in the previous paragraph.

LPS activation procedure. Five ng/mL LPS (E. coli 026:B6 LPS; Difco Laboratories, Detroit, MI) was added to each of four plastic test tubes. Fifty microliters of heparin (10 U/mL final concentration; Sigma, Oslo, Norway) was used as anticoagulant for 5.0 mL of drawn blood. The samples were then gently mixed, aliquoted into four test tubes, each containing 1.0 mL blood, and incubated at 37°C for 2 h under constant rotation at 200 rpm. The reaction was stopped using 100 μL of 2% EDTA, and samples were subjected to mononuclear cell isolation and blood plasma isolation.

Mononuclear cell isolation. One milliliter of 0.15 M NaCl was added to the blood samples, mixed well, carefully layered on top of Lymphoprep (Axis-Shield PoC AS, Oslo, Norway), and centrifuged at 420 × *g* (1500 rpm) for 15 min. The top layer was pipetted off and discarded, whereas the cell band was transferred to a new test tube containing 0.15 M NaCl and centrifuged at 1450 × *g* (2800 rpm) for 10 min. The liquid was removed and the resulting mononuclear cell pellet resuspended in 200 μL 0.15 M NaCl. All samples were then frozen at –20°C until quantification of tissue factor (TF) activity.

Quantification of TF activity. TF activity was measured from isolated monocytes and based on the ability of TF to accelerate Factor X activation mediated by Factor VIIa, using bovine Factor V and barium citrate eluate (containing Factor VII, Factor X, and Factor II) as described in previous studies (19).

Standard hematological parameters. Blood (5 mL) collected in EDTA was used for plasma isolation (4 mL), whereas 1 mL blood was transferred to a plastic test tube and allowed to stand undisturbed on the workbench for half an hour. Samples were then mixed well and cell counts were performed on a Sysmex K1000 automated hematology analyzer.

TNFα assay. TNFα was quantified using a human TNFα ELISA kit (CLB, Amsterdam, The Netherlands). The ELISA was based on a "sandwich-type" enzyme immunoassay using monoclonal antihuman TNFα antibody bound onto the polystyrene microtiter wells (to which the TNFα binds) and a second monoclonal TNFα antibody (biotinylated and to which the TNFα also binds). Horseradish peroxidase (HRP) conjugated streptavidin, which was bound to the biotinylated antibody, was used to react with a substrate, and the absorbance of the product was measured spectrophotometrically at 450 nm. Samples were diluted in a 1:20 ratio with dilution buffer. The assay sensitivity was 1–3 pg/mL.

Interleukin-8 (IL-8) assay. IL-8 quantification was obtained using a human IL-8 ELISA system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The ELISA was based on a highly specific IL-8 monoclonal antibody bound to the wells of a microtiter plate and a second polyclonal IL-8 antibody that is conjugated to HRP. A substrate is added, and the

TABLE 1
Content of EPA and DHA in the Various Products Tested in This Study, and the Daily Intake of EPA and DHA

Product[a]	Product (g/wk)	EPA (g/wk)	DHA (g/wk)	EPA + DHA (g/d)
Smoked salmon	400	3.31	5.00	1.20
Salmon fillet	400	3.17	5.04	1.20
Cod fillet	400	0.06	0.15	0.03
CLO	105	9.66	11.25	3.00

[a]Intervention with smoked salmon (400 g/wk), cooked salmon fillet (400 g/wk), cooked cod fillet (400 g/wk), or cod liver oil (CLO) (15 mL/d).

product is measured spectrophotometrically at 450 nm. Blood plasma samples were diluted in a 1:100 ratio with dilution buffer. The assay sensitivity was 10 pg/mL (range 1.56–100 pg/well).

Leukotriene B_4 (LTB_4) and thromboxane B_2 (TxB_2) assays. LTB_4 and TxB_2 levels in plasma of LPS-stimulated blood were determined using enzyme immunoassay (EIA) systems (Amersham Pharmacia Biotech). The EIA were based on the competitive binding of LTB_4 (TxB_2) and LTB_4 (TxB_2)-peroxidase to a rabbit anti-LTB_4 (TxB_2) antibody. The LTB_4 (TxB_2)-peroxidase reacts with 3,3′,5,5′-tetramethylbenzidine substrate to form a product that is measured spectrophotometrically at 450 nm. Therefore, sample LTB_4 (TxB_2) is inversely proportional to the concentration of unlabeled LTB_4 (TxB_2). Samples were diluted to a 1:6 ratio (LTB_4) and to a 1:60 ratio (TxB_2), respectively, with the assay buffer. The assay sensitivity for LTB_4 was around 0.3 pg/well or 6.0 pg/mL (range 0.3–40 pg/well), and the sensitivity for TxB_2 was approximately 0.2 pg/well (range 0.5–64 pg/well).

Statistical analyses. All results are presented as mean ± SD. Univariate and multivariate analyses were performed to search for significant changes caused by the various interventions. Within-group changes during the intervention period were analyzed using Student's *t*-test or Wilcoxon nonparametric signed rank test. Simultaneous, multiple comparisons of the test groups against the control group were performed using one-way ANOVA followed by Dunnett's *t*-test. Correlations were calculated by means of the Spearman's rank correlation coefficient (r). P values less than 0.05 were considered significant.

RESULTS

FA composition in the supplemented products. Table 1 shows the composition of n-3 FA in the various products (smoked salmon, cooked salmon fillet, cooked cod fillet, and CLO) used in this study. It is noteworthy that intake of salmon fillet was associated with almost 3 times less intake of EPA and DHA as compared with the group drinking 15 mL/d CLO.

The content of n-3 FA in serum after intake of fish and fish oils. One of the major interests of this study was to explore whether the source of n-3 FA influences the rise in their serum concentration after 8 wk of fish diet or fish oil supplementation. Thus, when consumption of 400 g of fish (salmon, smoked salmon, or cod fillet) per week was compared with the dietary supplementation of 15 g per day of CLO, FA analyses of serum content of EPA and DHA revealed a relatively high rise in n-3 FA in the salmon groups (Table 2). Although the intake of EPA and DHA was 3.0 g/d in the CLO group, the rise in total amount of n-3 FA in this group was not that much larger in EPA and even less marked in DHA when compared with the salmon fillet groups where the intake of EPA and DHA was 1.2 g/d. The rises in EPA and DHA were significantly larger in the CLO group and in both salmon groups compared with the changes in the cod and control groups ($P < 0.05$).

TABLE 2
EPA and DHA in Serum (mean values ± SD) Before and After 8 wk of Intervention[a]

Group	20:5n-3 (mmol/L) Before	20:5n-3 (mmol/L) After	Changes EPA/g consumed per day	22:6n-3 (mmol/L) Before	22:6n-3 (mmol/L) After	Changes DHA/g consumed per day
Smoked salmon	0.164 ± 0.072	0.288 ± 0.167[b]	0.26	0.269 ± 0.081	0.341 ± 0.132[c]	0.10
Salmon fillet	0.136 ± 0.068	0.312 ± 0.146[b]	0.39	0.261 ± 0.086	0.379 ± 0.157[c]	0.16
Cod fillet	0.195 ± 0.107	0.216 ± 0.123		0.311 ± 0.127	0.306 ± 0.116	
CLO	0.185 ± 0.152	0.381 ± 0.123[b]	0.14	0.285 ± 0.122	0.355 ± 0.068[c]	0.04
Control	0.227 ± 0.235	0.227 ± 0.221		0.271 ± 0.136	0.301 ± 0.164	

[a]Intervention with smoked salmon (400 g/wk), cooked salmon filet (400 g/wk), cooked cod filet (400 g/wk), or CLO (15 mL/d). The control group was not supplemented.
[b]Increase in EPA (20:5n-3) is higher in smoked salmon, salmon fillet, and CLO groups as compared with cod fillet and control groups ($P < 0.05$).
[c]Increase in DHA (22:6n-3) is higher in smoked salmon, salmon fillet, and CLO groups as compared with cod fillet and control groups ($P < 0.05$). For abbreviation see Table 1.

TABLE 3
White Blood Cells (WBC), Platelet Numbers, and Mean Platelet Volume (MPV) (mean values ± SD) Before and After 8 wk of Intervention[a]

Group	WBC (10^9/L) Before	WBC (10^9/L) After	Platelets (10^9/L) Before	Platelets (10^9/L) After	MPV (fL) Before	MPV (fL) After
Smoked salmon	5.90 ± 1.23	5.83 ± 1.46	250 ± 36	235 ± 33	9.63 ± 0.58	9.88 ± 0.58
Salmon fillet	6.03 ± 0.99	6.00 ± 1.24	255 ± 65	237 ± 47	9.74 ± 1.05	10.01 ± 1.15[b]
Cod fillet	6.37 ± 0.74	6.28 ± 1.82	257 ± 50	255 ± 55	9.37 ± 0.61	9.19 ± 0.48
CLO	6.24 ± 1.55	6.15 ± 1.49	260 ± 52	260 ± 52	9.61 ± 0.93	9.76 ± 1.09
Control	6.03 ± 2.24	6.58 ± 1.99	256 ± 71	252 ± 67	9.77 ± 0.96	9.65 ± 1.12

[a]Intervention with smoked salmon (400 g/wk), cooked salmon fillet (400 g/wk), cooked cod fillet (400 g/wk), or CLO (15 mL/d). The control group was not supplemented.
[b]Increase in MPV is higher in salmon fillet group as compared with the smoked salmon, cod fillet, CLO, and control groups ($P < 0.05$). For abbreviation see Table 1.

Changes in serum lipids. Intake of n-3 PUFA has been documented (7,8) to be associated with a reduction in TAG. In this study, however, only trends in TAG reductions were observed in all the groups except the control group, and no significant changes were observed in TAG, total cholesterol, or HDL-cholesterol (results not shown).

Changes in mean platelet volume (MPV) and cell counts. Among the blood cells, a significant increase in MPV was observed in the salmon fillet group as compared with MPV in the smoked salmon, cod fillet, and control groups (Table 3). MPV also tended to be increased in the smoked salmon fillet group and to a lesser degree in the CLO group (not significant). The rise in MPV was associated with a reduction in platelet count, but this was not significant. No significant changes were seen in any of the cell counts (white blood cells and platelets).

Changes in cellular activation products. No significant changes were observed in fibrinogen, tissue plasminogen activator, or plasminogen activator inhibitor-1 during the study (results not shown). LPS-stimulation of blood generates many activation products, which may be down-regulated by intake of n-3 FA. However, probably owing to the low number of subjects in each test group, there were no significant changes in these test parameters, although a trend was seen by the relatively small reduction of TNFα, LTB_4, and TxB_2 in all the groups excluding the cod fillet group (results not shown). Multivariate analyses revealed a negative correlation between changes in 20:5n-3, 20:6n-3, and changes in TNFα, IL-8, LTB_4, TxB_2, and TF ($P < 0.05$).

DISCUSSION

The major message of our study is that the apparent differences in sources of n-3 PUFA affect the content of EPA and DHA in serum lipids. After 8 wk on a diet consisting of n-3 FA-rich fish (smoked salmon or cooked salmon fillet) compared with supplementing the diet with CLO, the serum content of EPA and DHA was almost as high in the fish-eating groups as in the CLO group. The daily intake of EPA and DHA was almost 3 times less in the fish groups compared with the CLO group. Our results support the study by Visioli *et al*. (13), and revisited by Galli and Marangoni in 2006 (20), suggesting that a daily fish intake more effectively enhanced the plasma concentration of n-3 FA(s) as compared with intake of concentrated EPA and DHA ethyl esters in capsules. However, in the present study we show that consumption of as little as 2 meals per week including salmon (a total of 1.2 g EPA + DHA on average per day) is almost as effective as intake of 15 mL cod liver oil per day (a total of 3.0 g EPA + DHA per day) in raising the serum concentrations of EPA and DHA. This is experimental evidence that n-3 FA from fish are more effectively incorporated into serum lipids than when administered as a natural oil supplement. The administration of EPA as salmon fillet was nearly twice as effective compared with administration as CLO. Farmed salmon contains large amounts of TAG (93.2% of total lipid), and thus large amounts of EPA and DHA are bound to TAG whereas relatively small amounts of the FA are bound to phospholipids (21). In this regard, the distributions of EPA and DHA are similar in CLO, as they are all bound to TAG. Conceivably, the greater uptake of n-3 FA from fish as compared with CLO might be attributed to the more diluted emulsion of n-3 FA when consumed in the form of fish fillets as compared with the intake of a lipidic bolus of oil as CLO. This higher dilution together with their incorporation within the food item may favor the processes that are involved in fat digestion and absorption at the intestinal level. One may anticipate a higher surface of interaction between the food component and the intestinal wall together with a more favorable secretion of agents for the absorption of lipids.

A need of a nine times the amount (by wt) of DHA if served as capsules compared with to when served as salmon flesh was reported by Visioli *et al.* (13). An exaggerated incorporation of DHA from salmon is also seen in the present study, but it is not as pronounced as in the former work (13). This may reflect an enhanced effect when administered as TAG rather than as ethyl esters. DHA is predominantly esterified in the *sn-2* position whereas EPA is more randomly distributed in all three positions of TAG molecules (22). Since it has been reported that the absorption of EPA and DHA is higher when these FA are predominantly in the *sn*-2 position than when they are distributed randomly among the three positions of TAG molecule (23), this may at least partially account for the apparently greater incorporation of DHA from fish oil (CLO) as compared with the uptake from the concentrated EPA and DHA ethyl esters in capsules in the earlier work (13).The incorporation of

DHA is affected by the co-supplementation of EPA (23). A low ratio of EPA to DHA will probably favor the incorporation of DHA. In our study, this ratio was lower (0.9) than in the study by Visioli *et al*. (13), where the ratio was 1.4. In other words, EPA is more easily incorporated than DHA. When EPA/DHA is low, DHA incorporation is favored.

The incorporation of n-3 FA from cooked salmon fillets tended to be enhanced compared with the smoked salmon fillets (not significant). Differences in bioavailability that depend on food preparation methods and increased digestibility and bioavailability due to heat treatment are well documented (25).

It is well established that intake of EPA and DHA reduces serum levels of TAG. This is only observed as a trend in the present study and mainly in the salmon fillet and CLO groups, and surprisingly also in the cod fillet group.

In this study, intake of n-3 FA results in fewer but larger blood platelets; in particular, the intake of salmon results in larger MPV, but compared with the fish oil group the platelet number does not drop to the same extent. In a previous study we observed the following: Even if the whale oil contained less (than half of the) EPA and DHA compared with CLO, and the incorporation of these FA was lower in the group supplemented with whale oil compared with the group supplemented with CLO, the effect on or the increase in MPV was higher in the whale oil group than in the CLO group (26). This implies that the increased MPV is affected not only by a rise in n-3 FA but also by some unknown factors.

Intake of n-3 FA has been associated with reduction in cellular activation products of blood cells. In our model of LPS-stimulated whole blood, no significant reductions were found although TNFα, LTB_4, and TxB_2 tended to be lower after 8 wk in the salmon groups and CLO group. The failure to see significant reductions can be explained by the relatively strong agonist, LPS, used in our study, which may mask changes at the physiological level *in vivo*, i.e., activation reactions going on all the time. Larger numbers of subjects in each group would have been needed in this respect. Not surprisingly, multivariate analyses demonstrated that the changes in the inflammatory activation products showed a significant negative correlation to the changes in EPA + DHA.

Thermal processing of food produces both positive and negative effects, and the degree of heat-induced changes depends on the treatment applied (25). In fish processing, sarcoplasmic proteins, peptides, amino acids, and other water-soluble compounds are lost with drip (the fluid that is lost during handling), and cooking generally amplifies these losses (27). We failed to see any significant differences between the effects of cooked fish as compared with raw fish (smoked salmon). This may also be explained by the relative small groups in the present study and the lack of having a more strict diet regime for the test subjects.

It may be concluded that intake of fatty fish is more effective in increasing EPA and DHA than supplementation of diet with fish oils and is more likely to have beneficial effects on HDL-cholesterol and activation reactions of whole blood.

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Dietary Fish Oil n-3 Fatty Acids Increase Regulatory Cytokine Production and Exert Anti-inflammatory Effects in Two Murine Models of Inflammation

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ABSTRACT: The higher incidence of inflammatory diseases in Western countries might be related, in part, to a high consumption of saturated fatty acids and n-6 polyunsaturated fatty acids (PUFA) and an insufficient intake of n-3 fatty acids. The purpose of this study was to examine the effects of dietary n-3 fatty acids on innate and specific immune response and their anti-inflammatory action in models of contact and atopic dermatitis. Balb/C mice were fed for 3 wk either n-6 or n-3 PUFA-fortified diets. After inducing a contact or an atopic dermatitis, immunological parameters were analyzed to evaluate the anti-inflammatory potential of these n-3 PUFA. n-3 PUFA reduced innate and specific immune responses through inhibition of T_H1 and T_H2 responses, increase of immunomodulatory cytokines such as IL-10, and regulation of gene expression. The inhibition of both kinds of responses was confirmed by the anti-inflammatory effect observed in contact and atopic dermatitis. Reduction in weight, edema, thickness, leukocyte infiltration, and enhancement of antioxidant defenses in the inflamed ears of mice from both models along with the prevention of delayed-type hypersensitivity induced in atopic dermatitis proved n-3 PUFA efficacy. Our data suggest that dietary fish oil-derived n-3 fatty acids have immunomodulatory effects and could be useful in inflammatory disorders.

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The immune system is a complex set of interactive cells and molecules that has evolved to maintain the internal environment (1). Nutrition is one of the factors that regulate immune response and why a balanced and an adequate diet is required for optimal development and functioning of the immune system (2,3). Among different nutrients, lipids play a key role in immune function. Much of the interest in lipids has focused on polyunsaturated fatty acids (PUFA), especially those of the n-6 and n-3 families. The biological functions of these PUFA render them necessary for human well-being, especially because of their impact on nervous tissue development and immune function (4).

A possible explanation for the increase of arteriosclerosis, inflammatory, allergic, or autoimmune diseases, and cancer in Western countries may be related to the type of diets, plenty of saturated and *trans* fatty acids together with a high consumption of n-6 PUFA, and the absence of n-3 PUFA (5,6). The improvement reached with the Mediterranean diet, rich in oleic and n-3 fatty acids and scarce in saturated fats, confirms the relevant role of dietary lipids in human health, because of the low incidence of such pathologies (7). Experimental studies have provided evidence that incorporation of n-3 fatty acids into the diet also modifies inflammatory and immune reactions, making them potentially therapeutic for inflammatory and autoimmune response (8). In spite of the success in animal models (9–11), n-3 PUFA efficacy in humans is less conclusive (12,13).

Dietary fatty acids are able to modulate the immune system through several mechanisms that include alterations in lymphocyte proliferation, cytokine synthesis, phagocytic activity, or immune cell activities (14,15). Such modulation of immune functions may be produced as a consequence of several factors; but in general, the main event involved in this process is associated with changes in the cell membrane composition due to dietary fatty acid manipulation. However, phospholipid changes will depend on many factors such as the amount, type or time of dietary manipulation, and sex and species of animals (or humans) fed dietary lipids (16).

The main mechanism of action of n-3 PUFA is related to eicosanoids synthesis. When arachidonic acid (ARA) is substrate of cyclooxygenase (COX) or lipooxygenase (LOX) enzymes, eicosanoids with proinflammatory properties are produced, whereas EPA derived-compounds are less biologically potent. These intermediates, together with the competitive inhibition of ARA metabolism exerted by EPA, are the most important reasons to consider n-3 PUFA an anti-inflammatory compound (17). Other mechanisms of action are related to modulation of gene expression, involvement in signal transduction pathways, apoptosis or alterations of membrane fluidity, among others (4).

In this work we have analyzed the inflammatory response, innate and specific, in animal models of contact and allergic dermatitis after the consumption of an n-3 fortified diet in comparison with a common Western-like n-6 rich diet.

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Abbreviations: ARA, arachidonic acid; Con A, concanavalin A; COX, cyclooxygenase; DNFB, 2,4-dinitro-1-fluorobenzene; DTH, delayed-type hypersensitivity; FO, fish oil; GSH, glutathione; LOX, lipooxygenase; LPS, lipopolysacharide; LXR, liver X receptor; M-CSF, macrophage-colony stimulating factor; MDA, malondialdehyde; MPO, mieloperoxidase; PGE_2, prostaglandin E_2; PPAR, peroxisome proliferator-activated receptor; SO, sunflower oil.

EXPERIMENTAL PROCEDURES

Reagents. The fish oil used in de study was Eupoly®-EPA from Puleva Biotech, SA (Granada, Spain). All chemicals were of the highest available grade and were purchased from Sigma Chemical (St. Louis, MO, USA) except those indicated in the text.

Animals and diets. Weaned male Balb/C mice (n = 10 per group) of 3 weeks of age were purchased from the Granada University breeding colony and housed in a temperature (22ºC) and light (12 h) controlled cycle. This study was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (18), as promulgated by the National Institutes of Health, and it was approved by the Animal Research and Ethic Committee of the University of Granada (Spain). The global experiments were conducted three times with similar results.

Semisynthetic diets were prepared following the recommendations given by the American Academy of Veterinary for Animal Nutrition. Differences among the diets were only the source of fat included, which was 5% of the diet in all cases. Diets were composed of 19.1% crude protein, 5% crude oil, 5% crude fiber (in the form of α-cellulose), and 62.9% carbohydrates (which included 57.9% starch and 5% sucrose). Other components such as methionine and the mineral and vitamin mix represented 0.3% and 1%, respectively. After a methanol-toluene extraction, lipid profiles of the diets were obtained by gas chromatography coupled to flame ionization detection (FID) (Column SP2380 Supelco, 60 m × 25 mm) (9,19). In Table 1 diet lipid profiles are shown.

In each of the experimental models used in this work, animals were divided in two groups. One of the groups received a diet based on sunflower oil, rich in n-6 fatty acids (SO diet); the other group consumed a diet fortified with fish oil, rich in n-3 fatty acids (FO diet). Fish oil was supplemented at 15% of the fat content in the latter diet. Diets were consumed for 3 weeks and were conserved in argon atmosphere and at –80ºC. They were administrated daily to the mice to avoid PUFA oxidation.

During this period, weight, physical aspect, and behavior of the animals were evaluated. Animals were killed by intraperitoneal (IP) administration of sodium pentothal (50 mg/kg of body weight) and blood was collected by cardiac puncture in ethylenediamine-tetraacetic acid tubes. Blood was then centrifuged, 2,200 × g for 10 min at 4ºC, and plasma aliquots were collected and frozen at –80ºC.

Fatty acid contents in tissues. Total plasma, liver, and spleen fatty acid profile was determined as described (9,19). Livers and spleens were homogenized in distillated water (1:2) while plasma was directly used. After fat extraction lipid profile of tissues was determined in the same way as the lipid profile of diets.

Cell cultures. Spleens were removed from all mice and homogenized in complete culture medium, DMEM plus 10% FBS, plus 1% penicillin/streptomycin. After centrifugation (1,500 rpm, 5 min) erythrocytes were lysed with a lysing solution (NH_4Cl 1.7 mol/L, $KHCO_3$ 0.12 mol/L, ethylenediaminetetraacetic acid 9 mmol/L) for 15 min at 4°C. Resting cells were counted by using a hemacytometer and cultured to perform proliferation and stimulation assays in current culture medium (DMEM + 10% FBS). Cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere (20).

Bone marrow-derived macrophages were obtained from the mice and cultured in DMEM that contained 20% FBS and 30% L cell-conditioned media as a source of macrophage-colony stimulating factor (M-CSF) (21). After 7 days of culture, a homogeneous population of adherent macrophages was obtained. To render cells quiescent, at 80% confluence macrophages were deprived of L cell-conditioned medium for 16–18 h before the distinct stimulus (22).

Proliferation assays. Spleen-derived lymphocytes were cultured in 24-well plates (2×10^6 cells/well) in 1 mL of medium and stimulated with concanavalin A (Con A) (5 μg/mL) or lipopolysaccharide (LPS) (50 μg/mL). Bone marrow-derived macrophages were cultured in 24-well plates (1×10^5 cells/well) in 1 mL of medium and stimulated with L cell. Cell proliferation was measured by [^{3}H]thymidine incorporation as previously described (23,24).

Analysis of cytokine production, PGE_2, and histamine. Spleen-derived lymphocytes were cultured in 6-well plates (5×10^6 cells/mL) in 5 mL of medium and incubated during 24 h in

TABLE 1
Fatty Acid Composition of the SO and FO Diets[a]

		SO diet	FO diet
Oleic acid	C18:1 (n-9)	25.4	26.6
Linoleic acid	C18:2 (n-6)	51.4	41.7
α-Linolenic acid	C18:3 (n-3)	0.2	0.3
Arachidonic acid	C20:4 (n-3)	0.8	0.8
EPA	C20:5 (n-3)	0.24	1.45
DHA	C22:6 (n-3)	nd	1.42
Saturated fatty acids		20	22.8
MUFA		26.2	25.9
PUFA		53.7	51.2
n-6		45.5	39.3
n-3		0.63	3.04
n-3/n-6		0.01	0.08

[a]Values represent percentages (%) of total fatty acids. nd, Not detected.

TABLE 2
Cytokine and Nuclear Receptor PCR Primer Sequences

Gene	Forward	Reverse
IL-2	TGATGGACCTACAGGAGCTCCTGA	GAGTCAAATCCACAACATGCCGCA
IFNγ	TGGAGGAACTGGCAAAAGGATGGT	TTGGGACAATCTCTTCCCCAC
IL-10	TCCTTAATGCAGGACTTTAAGGG	GGTCTTGGAGCTTATTAAAAT
IL-1β	TGATGAGAATGACCTGTTCT	CTTCTTCAAAGATGAAGGAAA
IL-4	ACGAGGTCACAGGAGAAGGGAC	GGAGCAGCTTATCGATGAATCC
TNFα	AACTAGTGGTGCCAGCCGAT	CTTCACAGAGCAATGACTCC
PPARα	CTGCAGAGCAACCATCCGGAT	TTGTCTGAATCCTACTAGCC
PPARγ	ATGCCAAAAATATCCCTGGTTTC	CTTGGATGTCCTCGATGGGC
LXRα	GGGGCCAGCCCCCAAAATGCTG	GCATCCGTGGGAACATCAGTCG
LXRβ	GCAACGCTTTGCCCACTTCAC	GCGGCAGCTTCTTGTCCTGG
β-Actin	TGGAATCCTGTGGCATCC	AACGCAGCTCAGTAACAGTCC

the presence or absence of Con A (5 μg/mL) or LPS (50 μg/mL). The supernatants were collected after 48 h of incubation. Differentiated macrophages were cultured in 6-well plates (3×10^6 cells/well). After 12 h, adherent macrophages were stimulated with LPS (100 ng/mL). Supernatants were collected after 24 h. Concentrations of IgG_1, IgE, interleukin- (IL-) 2, IL-1β, IL-4, tumor necrosis factor alpha (TNFα), IL-12, IL-5, transforming growth factor beta (TGFβ), or interferon gamma (IFNγ) were determined by enzyme immunoassay (ELISA) according to the manufacturer's instructions (BETHYL, Montgomery, TX, USA, for immunoglobulins or CytoSets; BIOSOURCE, Camarillo, CA, USA, for cytokines) in plasma and supernatants from lymphocyte or macrophage cultures as indicated. Analysis of histamine (IBL Laboratories, Hamburg, Germany) and prostaglandin E_2 (PGE_2; Oxford Biomedical Research, Rochester Hills, MI, USA) levels in different samples were also determined by using the indicated commercial ELISA kits.

Analysis of gene expression by RT-PCR. Expression of IL-2, IFNγ, IL-10, IL-1β, IL-4, TNFα, peroxisome proliferator-activated receptor alpha (PPARα), PPARγ, LXRα, and LXRβ was analyzed. Total mRNA was isolated from tissue and cultured spleen cells by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Reversed transcription was performed by using Reverse Transcriptase AMV (Roche, Indianapolis, IN, USA). First-strand cDNAs were either stored at –20ºC or used directly in the PCR step. PCR reactions (25 μL total volume) were carried out as previously reported (25). Murine oligonucleotides primers used in the PCR were purchased from MWG-Biotech AG (Ebersberg, Germany) and the nucleotide sequences are shown in Table 2.

Measurement of malondialdehyde. Malondialdehyde (MDA) plasma concentration was measured by separation with HPLC as previously described (26), which is based on the thiobarbituric acid reaction and reverse-phase separation with fluorescence detection.

Induction of contact and atopic dermatitis and ear analysis. The contact dermatitis was induced in the left ear of each mouse by application of 80 μL of a solution including 0.5% of 2,4-dinitro-1-fluorobenzene (DNFB) in a mixture of acetone/olive oil (4:1 by vol) 12 h before they were killed. The right ear received the solvent alone (27).

In another group of animals, to induce an atopic dermatitis, mice were sensitized with 25 μL of 0.5% DNFB solution applied to the shaved dorsal skin. Five days later, 10 μL of 0.2% DNFB (a nonirritant dose) was applied onto both sides of the left ear and the same amount of solvent alone on the right

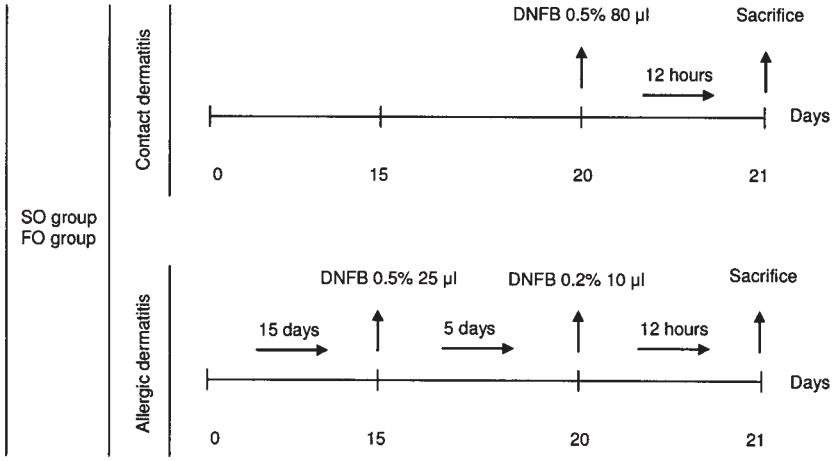

FIG. 1. Scheme of the animal models used.

ear (28). Figure 1 shows the designs of the two inflammatory models.

Both ears from animals of both studies were weighed. In the model of contact dermatitis, half of them were introduced in a heater for 24 h at 60ºC and afterward their dried weights and their consequent content of water were analyzed. In the model of atopic dermatitis, ear thickness was determined by a micrometer (Mitutoyo, Urdorf, Switzerland). The ears were homogenized in a 50 mmol/L solution of hexadecyltrimethyl-ammonium bromide to measure total glutathione (GSH), myeloperoxidase (MPO) activity, and cytokine levels. Such measurements were carried out following procedures described previouly (9,29).

Statistical analysis. All data are represented as mean ± SD values. Differences between means were tested for significance using one-way ANOVA. All statistical analyses were carried out with SPSS software (version 12.0; Chicago, IL, USA), with differences considered significant at $P < 0.05$.

RESULTS

Mice behavior and dietary intake of fatty acids. Animals were observed during the 3 wk the study lasted, and there were no significant differences in the average daily food intake between the two groups (4.0 ± 0.57 vs. 3.7 ± 0.91 g/day; SO vs. FO). Mice in the two groups grew normally, and there were no significant differences in weekly body weights (45.7 ± 29.9 vs. 52.5 ± 26.8 weight increment [%]; SO vs. FO, $P > 0.05$). No differences were observed between animals of the two experimental protocols (contact vs. allergic dermatitis) in spite of the initial abdominal DNFB dose at day 15 in the allergic model (data not shown). Moreover, no side effects such as occurrence of diarrhea or differences in either the aspect or the behavior were observed in the mice from the two groups.

The analysis of the diets confirmed the enrichment in n-3 fatty acids. Table 1 shows fatty acid compositions of the diets given. The approximate amounts of oleic acid, linoleic acid, EPA, and DHA ingested daily by mice fed the FO were 45 ± 8 mg, 87 ± 15.5 mg, 2.75 ± 0.5 mg, and 2.65 ± 0.5 mg, respectively, while SO-fed animals consumed 50.5 ± 7.1 mg, 102.5 ± 14.5 mg, and 0.5 ± 0.1 mg of oleic acid, linoleic acid, and EPA per day, respectively. Thus, the FO diet contained more than 10 times the amount of EPA and DHA n-3 fatty acids compared to the SO diet. This was the main difference between the diets since there were no important changes in other fatty acid profiles with the exception of n-6 PUFA content, which was slightly lower (45.5% vs. 39.3%; SO vs. FO). The increase in n-3 PUFA was compensated by a decrease in n-6 PUFA in the diet. Consequently, the n-3/n-6 PUFA ratio in the SO diet was clearly lower than that of the FO diet (0.01 vs. 0.08; SO vs. FO) (Table 1).

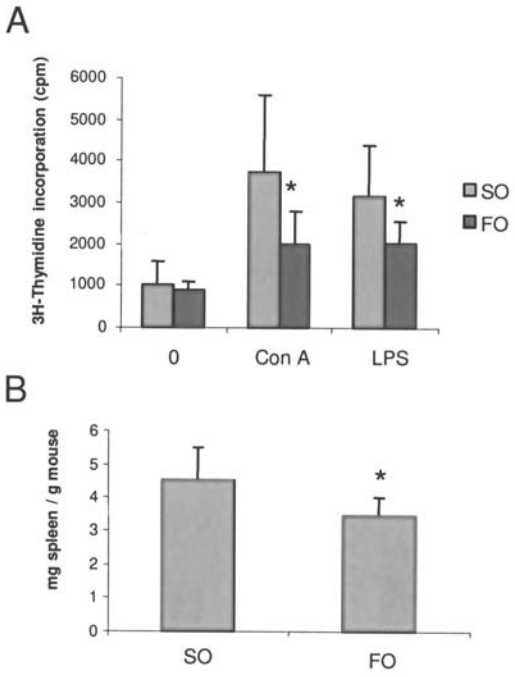

FIG. 2. FO diet decreases lymphocyte proliferation. (A) Spleen was removed from each mouse immediately after killing, erythrocytes were then lysed and spleen-derived lymphocytes from each mouse were cultured in the presence or absence of Con A (5 µg/mL) or LPS (50 µg/mL). [^{3}H]Thymidine (1 µCi/mL) was added and incubated for 48 h. Proliferation ratios were determined as [^{3}H]thymidine incorporation. Results are expressed as cpm ± SD ($n = 10$). (B) Milligrams (mg) of spleen per gram (g) of mouse body weight consuming SO or FO diets. Values are mean ± SD, $n = 10$. *Significant difference between SO and FO group ($P < 0.05$).

TABLE 3
Different Cytokines Measured by ELISA in Supernatants of Lymphocytes and Macrophages from Animals Consuming SO and FO Diets[a]

	Lymphocytes[b]		Macrophages[b]	
	SO diet	FO diet	SO diet	FO diet
IL-2	356.8 ± 191.2	200.1 ± 118*	nd	nd
IFNγ	42.6 ± 12.9	13.8 ± 5.1*	nd	nd
IL-1β	72.5 ± 29.9	38.6 ± 6.3*	690.4 ± 313.6	309.3 ± 44.2**
TNFα	368.5 ± 81.4	342.7 ± 109.8	5108.9 ± 1110	3950.8 ± 821.6*
IL-12	81.4 ± 19.8	68.08 ± 14.86	681.6 ± 295.9	679.6 ± 305.7
IL-4	965.1 ± 230.6	663.2 ± 272.7*	nd	nd

[a]Values are expressed in picograms per milliliter (pg/mL) and represent the mean ± SD (n = 10/group).
[b]*, **$P < 0.05$, $P < 0.01$, SO vs. FO group. nd, Not determined.

TABLE 4
Plasma T_H2 Cytokines and Allergy Mediator Levels

	SO diet	FO diet
IL-4	1.97 ± 0.65	1.74 ± 0.25[a]
IL-5	32.4 ± 2.6	25.7 ± 6.1
IgE	152.5 ± 120.4	78.8 ± 7.4[b]
IgG_1	75.2 ± 39.9	61.6 ± 23.6
Histamine	10.01 ± 2.6	3.03 ± 0.9[a]

[a]$P < 0.05$ vs. SO group.
[b]$P = 0.06$ vs. SO group.

FO diet reduces both T_H1 and T_H2 response. After 3 wk of diet consumption, proliferative response to mitogens of splenocytes was assessed by [^{3}H]thymidine incorporation (Fig. 2A). B and T cells obtained from FO-fed mice exhibited a significantly lower level of proliferation compared to cells from the SO group ($P < 0.05$ in both cases). In concordance with these findings, a reduction in the size of the spleen (Fig. 1B) was appreciated in the FO group.

To determine whether alterations in mitogen-induced IL-2 production by T lymphocytes might be responsible for the suppressive effects of dietary EPA and DHA fatty acids on lymphocyte proliferation, IL-2 secretion in the culture supernatants was measured. In this sense, T cells from FO-fed mice secreted less IL-2 relative to SO-fed mice when T cells were stimulated with Con A (Table 3).

Besides IL-2 reduction, other T_H1 cytokines were also modulated by the consumption of fish oil. In this sense, the levels of IL-1β and IFNγ produced by stimulated splenocytes were significantly lower when mice consumed the FO diet in contrast to the SO diet (Table 3). In the same way, we found lower levels of IL-12 in supernatants from stimulated FO-derived splenocytes although the results did not reach statistical significance.

The effects of the dietary lipids on macrophage proliferation induced by M-CSF were also evaluated, but there were no significant differences between the two groups (81,073 ± 15,574 vs. 91,530 ± 15,724 cpm; SO vs. FO). In spite of the absence of effect at this level, lower concentrations of inflammatory cytokines were found in the supernatants of cultured LPS-stimulated macrophages from FO-fed mice in comparison to SO-fed mice. In this sense, we observed reduction in the secretion of IL-1β and TNFα (Table 3) but IL-12 levels remained unchanged.

T_H2 parameters were also studied in mice fed with both diets. A decrease in the levels of IL-4 in supernatants obtained from spleen-derived lymphocytes was observed (Table 3). In a similar way, IL-4 concentration was also significantly lower in plasma (Table 4). Other T_H2 parameters were analyzed to check the impact of PUFA n-3 above allergic response, finding a decrease in other mediators such as IL-5, IgE, IgG_1, and histamine in plasma of the animals consuming the FO diet (Table 4).

The increase in immunoregulatory cytokines by the FO diet may be involved in the reduction of both T_H1 and T_H2 responses. Looking for an explanation for the immunomodulatory effects of the fish oil n-3 fatty acids in both T_H1 and T_H2 response, we studied the production of the regulatory cytokines

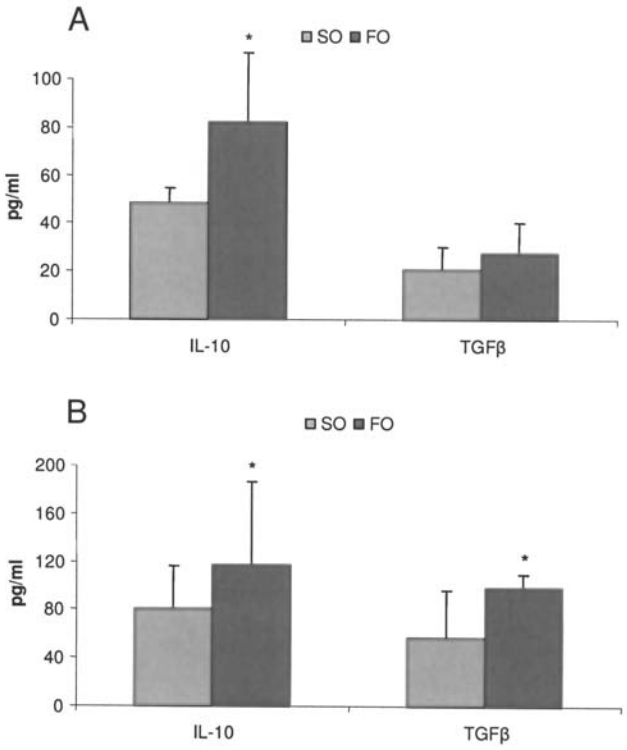

FIG. 3. n-3 fatty acids increase synthesis of immuneregulatory cytokines. (A) Spleen-derived lymphocytes from each mouse were cultured with or without Con A (5 μg/mL) as a T-cell stimulus. After 48 h in culture, supernatants were collected and IL-10 and TGFβ concentration was measured by ELISA. (B) Bone marrow-derived macrophages were cultured in the presence or absence of LPS (100 ng/mL). Production of IL-10 and TGFβ was measured. Results are expressed as mean ± SD (pg/mL). *Significant difference between SO and FO group ($P < 0.05$).

IL-10 and TGFβ, which are able to inhibit both responses in some circumstances. In this sense, levels of IL-10 in supernatants from stimulated T lymphocytes and LPS-stimulated macrophages were enhanced significantly in the FO group in contrast to the control (Fig. 3). Moreover, we also found higher amounts of IL-10 in serum of mice belonging to the FO group (24.58 ± 15.68 vs. 33.01 ± 10.5 pg/mL, SO vs. FO). Similarly, TGF-β production by both lymphocytes and macrophages was also increased in the FO group (Fig. 3).

Modulation of inflammatory eicosanoids and gene expression might be a mechanism of action of dietary n-3 PUFA. In vivo incorporation of dietary lipids was verified by liver, spleen, and total plasma lipids analysis (Table 5). In both tissues and plasma, fish oil significantly increased the content of EPA and DHA when compared to sunflower oil. There was more DHA than EPA in the analyzed samples, especially in the liver, despite that the content of EPA and DHA in the FO diet was similar. The increase in n-3 fatty acids in plasma and liver of FO-fed mice occurred without affecting the n-6 fatty acids, leading also to an increase in the n-3/n-6 ratio. However, in the spleen, the higher incorporation of EPA and DHA was associated with a decrease in n-6 PUFA, especially ARA, precursor of inflammatory eicosanoids.

TABLE 5
Lipid Profile of Plasma, Liver, and Spleen of Animals Fed the SO or FO Diet[a]

		Plasma[b]		Liver[b]		Spleen[b]	
		SO diet	FO diet	SO diet	FO diet	SO diet	FO diet
Oleic acid	C18:1 (n-9)	5.4 ± 1.4	8.9 ± 1.3*	23.1 ± 4.1	20.1 ± 2.7	11.5 ± 1.3	10.8 ± 1.5
Linoleic acid	C18:2 (n-6)	9.2 ± 3.8	15.2 ± 2.2*	19.6 ± 1.6	18.9 ± 0.4	10.3 ± 1.8	10.4 ± 0.9
α-Linolenic acid	C18:3 (n-3)	0.1 ± 0.1	0.1 ± 0.1	0.03 ± 0.01	0.04 ± 0.01*	0.3 ± 0.1	0.25 ± 0.02
Arachidonic acid	C20:4 (n-3)	3.9 ± 2.9	2.5 ± 2.8	8.7 ± 5.1	8.9 ± 1.3	13.5 ± 0.9	11.3 ± 0.5**
EPA	C20:5 (n-3)	0.1 ± 0.1	1.0 ± 0.3*	0.03 ± 0.01	0.4 ± 0.1*	Nd	0.56 ± 0.1**
DHA	C22:6 (n-3)	0.5 ± 0.2	3.5 ± 0.6*	1.6 ± 0.3	8.4 ± 1.7*	1.3 ± 0.2	6.0 ± 0.3**
Saturated fatty acids		77.9 ± 9.7	61.5 ± 6.8*	32.5 ± 6.1	34.8 ± 1.5	33.3 ± 0.8	38.2 ± 5.8
MUFA		8.5 ± 2.7	14.2 ± 3.6*	35.0 ± 7.3	26.6 ± 5.2*	12.5 ± 1.5	12.5 ± 1.7
PUFA		13.5 ± 7.2	24.2 ± 5.2*	32.4 ± 3.7	38.6 ± 3.7	26.5 ± 0.8	27.0 ± 5.4
n-6		11.1 ± 6.4	14.8 ± 2.0	28.6 ± 3.8	27.6 ± 2.1	25.3 ± 0.9	21.6 ± 4.7*
n-3		0.7 ± 0.5	7.6 ± 3.3*	2.5 ± 0.4	9.3 ± 1.8*	1.6 ± 0.3	6.8 ± 0.3**
n-3/n-6		0.1 ± 0.1	0.5 ± 0.2*	0.09 ± 0.02	0.3 ± 0.05*	0.06 ± 0.01	0.3 ± 0.08**

[a]Values represent percentages (%) of total fatty acids and data are expressed as mean ± SD (n = 10/group).
[b]*, **$P < 0.05$, $P < 0.01$, SO vs. FO group. nd, Not detected.

These data suggest that the release of eicosanoid mediators from the immune cells might have been affected by the FO diet. To check this hypothesis, we analyzed the n-6 fatty acid-derived eicosanoid PGE_2 in splenocytes and in macrophage supernatants. The results obtained showed lower concentrations of this inflammatory mediator produced by lymphocytes of the FO-fed mice (4.1 ± 0.2 vs. 2.4 ± 0.9 ng/mL, SO vs. FO; $P < 0.05$) or produced by stimulated macrophages (8 ± 0.2 vs. 5 ± 0.6 ng/mL, SO vs. FO; $P < 0.05$).

Due to the different possibilities of action of fatty acids, it was decided to investigate other possible mechanisms involved in the immunomodulatory effects observed for fish oil-enriched diets.

Dietary fatty acids are known for their capacity for modulating gene expression. When we analyzed gene expression after n-3 PUFA consumption, we observed an increase of IL-10 expression in T lymphocytes (Fig. 4A). The decrease of inflammatory and allergic cytokines observed was also associated with a lower expression of the corresponding genes (Fig. 4A, B). Moreover, the expression of other proteins involved in immune response was altered. In this sense, the expression of nuclear factors such as LXRα, LXRβ, or PPARγ was increased by the FO diet, suggesting its anti-inflammatory action (Fig. 4C). However, PPARα expression was not affected by fish oil consumption.

Other mechanisms suggested were those related to the effects carried out by products of lipid peroxidation of n-3 PUFA. However, we found similar levels of MDA in serum from mice consuming SO or FO diets (1.3 ± 0.1 vs. 1.4 ± 0.3 μM, SO vs. FO), suggesting that an increase in lipid peroxidation is not responsible for the effects of the different diets.

Since macrophage and lymphocyte responses were affected by dietary fish oil, immune disorders mediated by these could be prevented by n-3 PUFA consumption. To check the capacity of n-3 fatty acid of suppressing these kinds of responses, we evaluated the FO diet in animal models of contact and allergic dermatitis in which innate or specific responses are involved, respectively (30).

Prevention of contact dermatitis by the supplementation with fish oil n-3 fatty acids. To confirm the results we checked

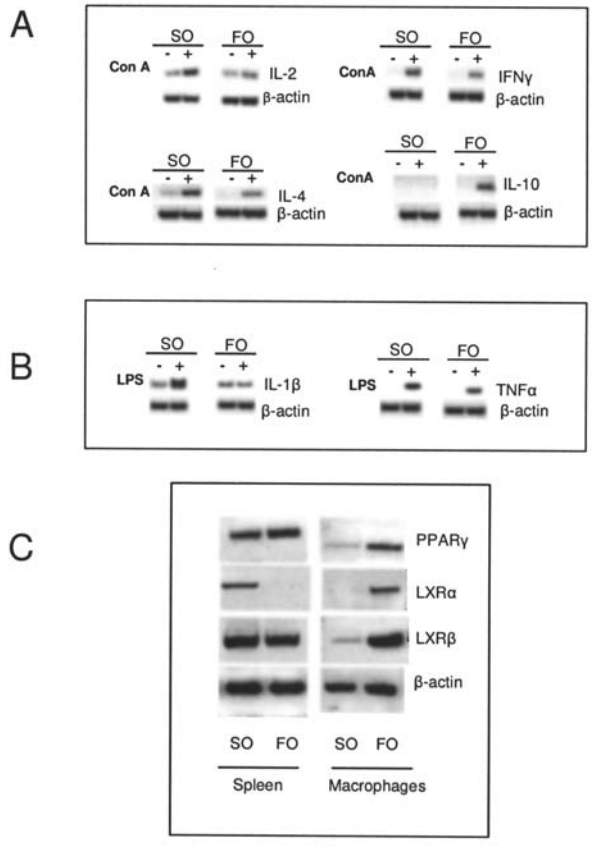

FIG. 4. FO diet alters expression of genes related to immune function. (A) Total mRNA from control and Con A-stimulated T lymphocytes was obtained and expression of IL-2, IFNγ, IL-4, and IL-10 was analyzed by reverse transcription (RT)-PCR. (B) mRNA from control and LPS-stimulated macrophages was extracted and the expression of TNFα and IL-1β was evaluated. (C) LXR·, LXRβ, and PPARγ expression in lymphocytes and macrophages was studied by RT-PCR. β-Actin was used as a control.

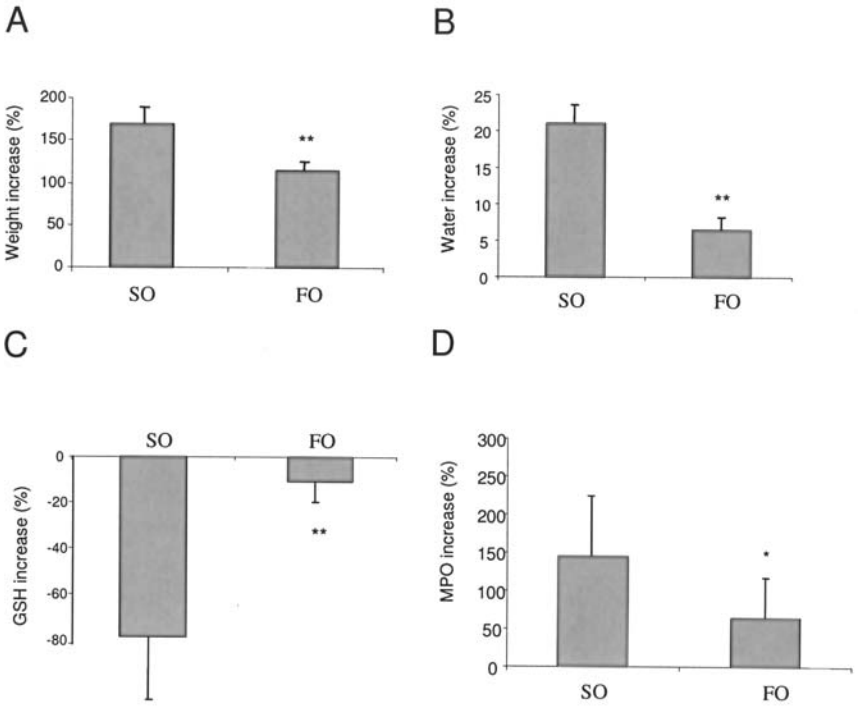

FIG. 5. FO diet improves contact dermatitis. (A) Percentage of weight increase of treated ears compared to healthy ones in mice consuming SO or FO diets. (B) Percentage of water increase of treated ears compared to healthy ones in mice consuming SO or FO diets. (C) Percentage of glutathione increase in treated ears compared to healthy ones in mice consuming SO or FO diets. (D) Increase of myeloperoxidase activity in treated ears compared to healthy ones expressed as percentage in mice consuming SO or FO diets. Results are expressed as mean ± SD ($n = 10$). **Significant difference between SO and FO groups ($P < 0.01$).

the effects of the diets in a murine model of ear contact dermatitis. Once the contact dermatitis was induced, ears were removed and weighed. As a consequence of the inflammatory process, ear weight increased in both groups; however, FO-fed mice showed a significantly weaker increase than SO-fed mice (Fig. 5A).

To determine the severity of the edema, the content of water in the ears from mice were measured. Differences between healthy and inflamed ears showed a higher increase of extravased liquids in ears from SO-fed mice in contrast to FO-fed mice (Fig. 5B). Moreover, the reduction of GSH in the inflamed ears in comparison to the healthy ones was lower in FO-fed mice, suggesting a protective role of the dietary n-3 fatty acids against oxidative stress during inflammation (Fig. 5C). Along with these results, we also observed a decrease in leukocyte infiltration measured as MPO activity in FO-fed mice, indicating that the FO diet improved the course of the inflammation (Fig. 5D). Finally, the lower local production of TNFα (99.8 ± 18.6% vs. 41.2 ± 7.3% of TNFα increase, SO vs. FO; $P < 0.05$) in the irritated ears of mice consuming FO diet confirmed the anti-inflammatory effects of n-3 fatty acids in this model of acute inflammation. Other cytokine concentrations were determined, but no changes were found (data not shown).

Prevention of atopic dermatitis by the supplementation with fish oil n-3 fatty acids. To better evaluate the lymphocyte involvement in the inflammatory response and the n-3 PUFA effects, we explored the anti-inflammatory actions of n-3 fatty acids on a second model of dermatitis that involves an allergic reaction and consists of two doses of DNFB (see experimental procedures section). After the first sensitization dose in the abdomen, we observed an absence of the delayed-type hypersensitivity (DTH) in animals consuming the FO diet (Fig. 6) in contrast to animals belonging to the SO group, in which the DTH appeared 3 days after the first challenge. The analysis of

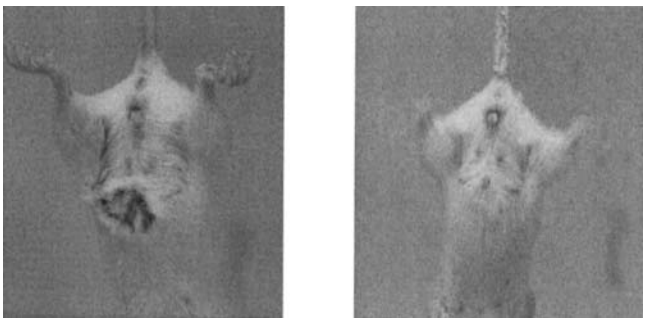

FIG. 6. Dietary fish oil prevents delayed-type contact dermatitis (DTH). (A) DTH occurred in mice belonging to the SO group after the first sensitization in the abdomen with DNFB (0.2%, 25 µL). (B) DTH was absent in mice belonging to the FO group.

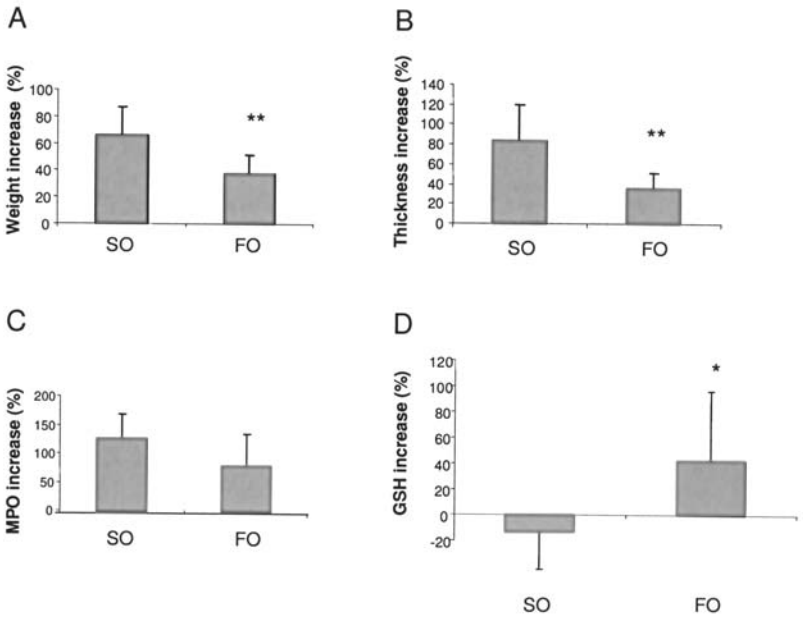

FIG. 7. FO diet improves allergic dermatitis. (A) Percentage of weight increase of treated ears compared to healthy ones in mice consuming SO or FO diets. (B) Percentage of thickness increase of treated ears compared to healthy ones in mice consuming SO or FO diets. (C) Percentage of glutathione increase in treated ears compared to healthy ones in mice consuming SO or FO diets. (D) Increase of myeloperoxidase activity in treated ears compared to healthy ones expressed as percentage in mice consuming SO or FO diets. Results are expressed as mean ± SD (n = 10). Significant difference between SO and FO groups (*$P < 0.05$, **$P < 0.01$).

the ears at the place of the second sensitization revealed an improvement of the inflammatory process induced. Reduction of ear swelling occurred after n-3 PUFA intake as was observed from the lower weight and thickness reached by irritated ears in mice from the FO group (Fig. 7A and B). GSH concentrations in damaged ears were not only restored but also increased (Fig. 7C); whereas, in the same way as in the model of contact dermatitis, MPO activity was decreased in ears of FO-fed animals (Fig. 7D). Local production of cytokines was also analyzed and only IL-4 levels were significantly decreased by dietary fish oil in irritated ears (153.1 ± 83.3 vs. 107.5 ± 26.2 pg/mL, SO vs. FO; $P < 0.05$).

DISCUSSION

A series of studies have demonstrated that EPA and DHA n-3 PUFA from fish oil have many biological effects, ranging from decreasing the levels of serum triacylglycerols (31) and reducing blood pressure (32) to inhibiting the growth of tumor cells (33), and modulating symptoms in patients with autoimmune and inflammatory diseases (34). The importance of n-3 essential fatty acids in the diet is consequently evident as well as the need to return to a more physiological n-6/n-3 ratio of about 4:1 rather than a ratio of 20–30:1 provided by current Western diets. Because of the relevant role of n-3 fatty acids, the scientific community is trying to explain their anti-inflammatory effect, to have scientific proofs that lead people to increase fish oil-derived n-3 fatty acids consumption. In this sense, it is essential to elucidate the mechanisms by which dietary fish oil suppress the immune response to maximize the potential therapeutic anti-inflammatory effects without compromising important antimicrobial or anti-tumor functions of the immune system.

The suppressed T- and B-lymphocyte proliferative response observed in FO-fed mice is consistent with the results of other investigations regarding fish oil (35,36). As IL-2 is a potent polyclonal autocrine and paracrine T-lymphocyte growth factor, the reduced IL-2 secretion by lymphocytes found in the FO-fed mice suggests that the suppressed lymphocyte proliferation observed was due, at least in part, to a reduction in IL-2, as has previously been shown (36).

As a result of the decrease in proliferation, other lymphocyte responses could also be affected. In this way, n-3 fatty acids not only were able to reduce IL-2 production, but also other T_H1 and T_H2 cytokines. In addition, the production of IL-1β and TNFα by macrophages was also decreased, indicating that diets supplemented with n-3 fatty acids have the capability to alter the whole cytokine profile. In spite of the strong effect of n-3 PUFA on inflammatory cytokines, they hardly modulated the secretion of the more characteristic T_H1 cytokine IL-12 by both macrophages and lymphocytes, suggesting that the inhibition of the inflammatory response does not imply a lower capacity of response to antigens. As this cytokine has important implications for host infectious disease resistance, the maintenance of its level at normal amounts suggests a correct function of the organism defenses. This is in contrast to considerations by other authors stating that n-3 fatty acids lead to an immunosuppressive status (37).

The decrease in cytokines exerted by these lipids could be related to the immunosuppressive effect regulated by IL-10 and TGFβ, which levels appear to be increased in macrophages and lymphocytes from the FO group. Despite that other groups showed the same increase of IL-10 after fish oil ingestion, none of these authors considered this immunoregulatory cytokine responsible for immune suppression (38). To confirm this hypothesis, research with IL-10–/– knockout mice would be necessary.

Although in this work we have not established the mechanism directly responsible for the observed cytokine modulation, several mechanisms could be proposed, namely eicosanoid modulation and gene expression modulation.

The reduction of eicosanoid production of series 2 and 4, such as PGE_2, by immune cells is indicative of the incorporation of n-3 fatty acids in membrane phospholipids. In fact, the results from this study demonstrate the significant increase of EPA and DHA in plasma, liver, and spleen (Table 5). Both fatty acids were incorporated in tissues substituting, in the case of spleen, ARA. This result might explain the lower levels of arachidonic-derived eicosanoids found. Although quantities of both fatty acids were similar in the diets, DHA is significantly enhanced, especially in liver samples, which indicates that, possibly, part of the EPA is converted into DHA by elongases and desaturases, important enzymes of lipid metabolism (3). The higher levels of DHA in tissues are logical, as DHA is the major form of restoration of n-3 PUFA in the organism (39). However, the reason immunoregulatory cytokines are increased by immune cells is not related to PGE_2, although other arachidonic-derived eicosanoid may be involved, since the lower levels of PGE_2, a potent inductor of IL-10, should lead to reduced concentrations of IL-10 (40).

Another potential mechanism that may explain the results described in this document is the capacity of n-3 PUFA to modulate gene expression, a mechanism by which these fatty acids could reduce inflammatory/T_H1 and T_H2 cytokines or increase T_H3 cytokines. The lower expression of several cytokines induced by fish oil consumption could be the consequence of a direct effect of the n-3 fatty acids on the implicated genes or an indirect effect exerted by IL-10, a cytokine capable of reducing the expression of inflammatory cytokines (41). In this sense, recent studies have demonstrated the capacity of certain PUFA to directly suppress Nuclear factor κB activation in immune cells through an ERK-mediated IL-10 mechanism, leading to an anti-inflammatory effect (42). In a similar way, higher expression of PPARγ and LXR in immune cells leads to a more potent anti-inflammatory effect as they mediate anti-inflammatory events (43). It is noteworthy that the observed PPARγ protective role in several diseases seems to be partly independent of IL-10 (44), a fact that indicates that the anti-inflammatory effect of n-3 PUFA may be due to the combination of the increase in IL-10 and the higher expression of PPARγ. In fact, EPA and DHA have been described to activate these nuclear receptors (45).

From the results obtained in healthy animals we could extrapolate the important role of dietary n-3 PUFA in both natural and acquired responses and, consequently, their potential therapeutic applications. To demonstrate their therapeutic role, we checked how a contact dermatitis, characterized by an innate response, mainly mediated by local macrophages and neutrophils, and an allergic dermatitis, with a specific lymphocyte response involved, could be improved by intake of diets enriched with fish oil.

The contact dermatitis induced resulted in an acute inflammation of the skin characterized by the two main features of the process, swelling (edema) and granulocyte infiltration, but does not seem long enough to induce a clear T_H1 or T_H2 response, which needs at least 24 h to develop. Although there are limitations in the use of this model for inflammatory reactions, it is one approach for studying the initial phase of inflammation (46) and could give some clues about the anti-inflammatory potential of n-3 PUFA. In this model, weight and water content of irritated ears was lower in FO-fed mice, suggesting that vasodilatation and edema had been less drastic. The weaker leukocyte infiltration could be explained by a lower MPO activity in the ears of these mice. The amelioration of this key step during inflammation, together with reduction of TNFα in the damaged ears by n-3 fatty acids, points to a critical role of these n-3 PUFA to attenuate the inflammatory process.

As a result of the irritation induced, a drastic decrease of antioxidant defenses took place, and GSH levels in the irritated ears were reduced. However, the reduction in GSH observed in inflamed ears from the FO-fed mice was less severe, possibly because of a lower breakdown of GSH or because of a better capacity to restore antioxidant defenses. Restoration of GSH after n-3 PUFA consumption in inflammation models correlates with effects observed in other studies (47). In fact, the possible antioxidant effect of n-3 PUFA has been already suggested (48), a fact that correlates with the similar plasma levels of MDA found in mice consuming, or not, the n-3 PUFA diet.

The model of allergic dermatitis, also called contact hypersensitivity, implies, however, complete T_H1 and T_H2 responses and allows a more exhaustive study of the n-3 fatty acids effects in inflammation. In addition, allergic response can also be studied since a type IV allergy takes place in this model (49).

Contact hypersensitivity is a T cell-mediated cellular immune response caused by repeated epicutaneous exposure against contact allergens, chemically reactive haptens that are able to bind directly to soluble or cell-associated proteins. This reaction is divided into two phases, sensitization and elicitation. In the first phase allergens are presented to naïve T cells, and in the second phase allergen-specific T cells are activated upon rechallenge with the same allergen, resulting in local inflammation (50). Both acute and T cell-mediated responses were prevented with the prophylactic administration of dietary n-3 PUFA.

In this sense, the results from our study demonstrate the capability of n-3 PUFA to suppress a specific immune response. On one hand, DTH after the first challenge was totally avoided in FO-fed mice, suggesting the n-3 PUFA-suppressive action on T lymphocytes and especially macrophages, the major type of cells involved. This effect attributed to n-3 PUFA has been

previously suggested by other groups (51). On the other hand, local inflammation was also ameliorated in the same way as in the previous model. Thus, leukocyte infiltration, ear swelling, and GSH content of the treated ears improved.

Such a model of inflammation mimics atopic dermatitis in humans and can curse as a T_H1 or a T_H2 response depending on the situation. The reduction of IL-4 concentration observed in ears suggests the capacity of n-3 PUFA to downregulate the T_H2 response induced, collaborating with the restoration of the damaged tissues. On the contrary, in the contact dermatitis model, inflammatory cytokines such as TNFα were decreased by fish oil; whereas in this model, the T_H1 local response is not so important in the elicitation phase. This fact, together with the prevention of DTH suggests an antiallergenic effect for n-3 PUFA. Other authors have already suggested an important role of n-3 PUFA in allergic sensitization and allergic pathologies (52), and how changes in Western diets have contributed to enhance incidence of these kinds of disorders (5). A more complete study of the systemic response should be carried out to clarify the role of n-3 PUFA during the different phases of the model.

In conclusion, we can attribute to fish oil-derived n-3 fatty acids an anti-inflammatory role with several possible therapeutic applications, especially in autoimmune and chronic inflammatory diseases. In an attempt to elucidate the mechanisms of action, immunoregulatory cytokines and special mediators such as nuclear receptors seem to be involved through their immunosuppressive actions. However, additional studies are required to further clarify the mechanisms of immunomodulation to optimize the potential beneficial effects of EPA and DHA.

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Long-Chain n-3 Polyunsaturated Fatty Acid Incorporation into Human Atrium Following Fish Oil Supplementation

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ABSTRACT: Recent studies have demonstrated that long-chain n-3 PUFA (LCn-3PUFA) are beneficial in reducing the risk of cardiac arrhythmias. This study was conducted to determine the extent of incorporation of LCn-3PUFA into human atrium following supplementation with a fish oil concentrate high in LCn-3PUFA. Volunteers preparing for coronary bypass surgery were randomized either to the treatment group (n = 8), receiving 6 g/d of fish oil concentrate (4.4 g of LCn-3PUFA), or the placebo group (n = 9), receiving 6 g/d of olive oil for a minimum period of 6 wk. Blood samples were collected prior to commencement of treatment, and preoperatively before bypass surgery. Atrial biopsies were obtained during surgery. The plasma and atrium samples were analyzed by GC following trans-methylation to determine FA profile. Post-supplementation, the treatment group had significantly higher plasma levels of 20:5n-3, 22:5n-3, and 22:6n-3 than the placebo group. Analysis of the atrium total lipids revealed a significant increase in the proportion of 20:5n-3 following fish oil supplementation. There was no significant difference in the concentration of 22:5n-3 and 22:6n-3 in the atrium total lipids; however, an upward trend was observed in subjects receiving fish oil supplementation. In the phospholipid fraction of the atrium, both 20:5n-3 and 22:6n-3 increased, whereas 20:4n-6 levels decreased. This study demonstrates for the first time that short-term supplementation with fish oil concentrate results in significant incorporation of LCn-3PUFA with a concomitant depletion of the eicosanoid substrate (20:4n-6) in the human atrium.

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Observational, clinical, and experimental studies have demonstrated that long-chain n-3 PUFA (LCn-3PUFA) supplementation reduces the risk of heart diseases including cardiac arrhythmias (1,2). Several mechanisms by which LCn-3PUFA prevent cardiac arrhythmias have been proposed. Studies using experimental animals have shown that LCn-3PUFA supplementation prevents arrhythmias by increasing the ventricular fibrillation threshold (3,4) and by decreasing ventricular fibrillation (5,6). Calo *et al.* (7) demonstrated that short-term LCn-3PUFA administration in patients undergoing coronary artery bypass graft (CABG) substantially reduced the incidence of postoperative atrial fibrillation and was associated with a shorter hospital stay. Other human studies have confirmed anti-arrhythmic potential of LCn-3PUFA (7–9). In isolated cardiac myocytes, EPA, and DHA have been shown to reduce contraction (10,11) and ouabain-induced arrhythmias (12). Several mechanisms have been suggested to explain the anti-arrhythmic actions of LCn-3PUFA, and they have been extensively reviewed (13–15). Some of the mechanisms suggested include modification of membrane phospholipids, alterations in the type and amount of eicosanoids generated, and the effects of these modifications on intracellular calcium release and calcium channels via the second messenger operated signaling system.

Billman *et al.* (5) suggested that non-esterified LCn-3PUFA have a direct antiarrhythmic effect without prior incorporation of LCn-3PUFA into myocardial phospholipids. However, as arrhythmias occur suddenly it is impractical to infuse non-esterified LCn-3PUFA. Evidence suggests that the incorporation of LCn-3PUFA into myocyte membrane phospholipids is essential for anti-arrhythmic action, so that these FA are readily available for release as free acids to prevent arrhythmias following myocardial ischemia (15). Indeed, phospholipase activity has been shown to be increased following myocardial ischemia (16). Regardless of the mechanism by which LCn-3PUFA supplementation prevents cardiac arrhythmias, incorporation of LCn-3PUFA into the myocardium pools appears to be a prerequisite for their action (15). In the animal experiments very high doses of dietary LCn-3PUFA supplementation were used. It is not known if dietary supplementation of LCn-3PUFA practically achievable with fish oil capsules in humans will result in significant incorporation of these FA into cardiac membranes.

Therefore it was of interest to investigate whether LCn-3PUFA supplementation results in the enrichment of atrium with LCn-3PUFA. Patients scheduled to undergo coronary bypass surgery were supplemented with fish oil capsules prior to obtaining blood and atrium biopsies. It was assumed that LCn-3PUFA incorporation into the lipid fractions of atrium biopsies would reflect LCn-3PUFA enrichment of the ventricle tissue. Because mechanisms for anti-arrhythmic action of LCn-3PUFA may be mediated via changes in the FA composition of cardiac membrane phospholipids and/or amount and type of LCn-3PUFA in the non-esterified fraction (15), FA composition of atrium major lipid fractions was determined separately.

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EXPERIMENTAL PROCEDURES

Subjects. Patients attending preoperative clinic for coronary artery bypass graft requiring a left internal mammary artery graft to left anterior descending artery were screened for the study. Patients were randomized 6 wk prior to the surgery to receive either a fish oil (Lube AS, Hadsund, Denmark) concentrate (six 1-g capsules, each containing approximately 676 mg of EPA and DHA, providing a total of approximately 4.4 g/d of LCn-3PUFA, $n = 9$) or a placebo (six 1-g olive oil capsules, $n = 8$).

The study protocol was approved by the Human Research Ethics Committee of the University of Newcastle and Hunter Area Research Ethics Health Committee, and the subjects gave informed consent prior to randomization. Characteristics of the patients involved in the trial are presented in Table 1. There was no significant difference in the mean age of the placebo and the treatment groups. All the patients except one in the placebo group were males. A large majority of the patients (with the exception of one in the fish oil group) were taking aspirin prior to the surgery. One participant in each of the placebo and the fish oil group was on ACE inhibitors, and nearly half of them in each group were taking statin drugs as a cholesterol-lowering therapy.

FA composition of the placebo and fish oil concentrate capsules is presented in Table 2. The fish oil concentrate contained 73.5% long-chain LCn-3PUFA in the form of 20:5n-3 (37.5%), 22:5n-3 (5.9%), and 22:6n-3 (30.1%). Six fish oil capsules, therefore, supplied about 4.3 g/d of LCn-3PUFA. The placebo oil was devoid of these FA and contained saturated (12.1%), monounsaturated (81.9%), and n-6PUFA (5.0%).

The supplements continued until the day prior to the surgery. Compliance was measured by the capsule count-back method and by LCn-3PUFA incorporation into plasma lipids. Baseline and preoperative blood samples were collected for measurement of cholesterol, triglyceride, LDL cholesterol, HDL cholesterol, and FA analysis. Atrium biopsies were obtained during the surgical procedure, frozen in liquid nitrogen, and stored at –80°C.

FA analysis. The FA composition of the plasma lipids was determined according to a modification of the method of Lepage and Roy (17). Briefly, 100 µL of plasma was mixed with 2 mL of methanol:toluene (4:1 vol/vol) containing 4 µg/mL of C21:0 as an internal standard, and 0.12 mg/L butylated hydroxytoluene as an antioxidant, in a glass culture tube, and vigorously mixed by vortex. This was followed by the slow addition of 200 µL of acetyl chloride while mixing by vortex. The tube was then sealed with a teflon-lined cap, and heated at 100°C for 60 min. The tubes were then rapidly cooled under running water. The reaction was halted by the addition of 5 mL of 6% K_2CO_3 solution, followed by vigorous mixing by vortex. The tubes were then centrifuged at $3,000 \times g$ for 10 min. at 4°C. Following centrifugation, the toluene supernatant containing the FAME was transferred to a glass vial and crimp sealed with a teflon-lined cap for subsequent analysis by GC.

FAME were quantified using a Hewlett Packard 6890 gas chromatograph equipped with a 30 m × 0.25 mm capillary column (DB-225, J&W Scientific, Folsom, CA) and a flame ionization detector (18). Chromatographic conditions were as follows: inlet temperature 250°C, inlet pressure 97.5 kP, split ratio 20:1, carrier gas as hydrogen (ultra-high purity) with a flow rate 1.6 mL/min. The chromatographic conditions were as follows; initial temperature of 170°C held for 2 min, ramped to 190°C at a rate of 10°C/min, held there for 1 min, ramped to 220°C at a rate of 3°C/min, and held at 220°C for 15 min. FAME were identified and quantified by comparison with authentic FAME standards (Nu-Chek-Prep, Elysian, MN)

Lipids from the atrium tissues were extracted by the methods described by Folch *et al.* (19). An aliquot of the total lipid extract was methylated and FA composition determined as described above. The remaining lipid extract from the atrial tissues was spotted on TLC plates, and the plates were developed in a solvent system comprising hexane:diethyl ether:acetic acid (85:15:1, by vol) for the separation of phospholipid, triglyceride, and non-esterified fractions (20). Lipid fractions were scraped off the plates and methylated, and FA composition was determined as detailed above.

Statistical analysis. The results are presented as mean ± SD.

TABLE 1
Characteristics of the Study Participants

	Placebo group ($n = 9$)	Fish oil group ($n = 8$)
Average age (y)	60.1	61.6
Gender (M/F)	8/1	8/0
Aspirin	9	7
Diuretics	1	2
ACE inhibitors	1	1
Statins	5	4

TABLE 2
FA Composition of Placebo and Fish Oil Concentrate (%, wt/wt) Determined by GC

FA	Placebo[a]	Fish oil[a]
14:0	0.0	0.4
16:0	8.2	2.3
16:1n-7	0.6	2.8
18:0	3.8	1.1
18:1n-9	79.1	9.0
18:1n-7	2.2	2.2
18:2n-6	4.4	1.5
18:3n-6	0.6	1.0
18:3n-3	0.5	0.1
20:3n-6	0.0	0.4
20:4n-6	0.0	2.7
20:5n-3	0.1	37.5
22:5n-3	0.0	5.9
22:6n-3	0.0	30.1
24:1n-9	0.0	0.2
Total SFA	12.1	3.8
Total MUFA	81.9	14.2
Total n-6 PUFA	5.0	5.5
Total n-3 PUFA	0.6	73.6

[a]Three random placebo and fish oil concentrate capsules were analyzed, and the average ($n = 3$) values are presented in the table.

Prior to analysis, data were assessed for linearity and homoscedasticity assumptions using the Scatterplot option of the Statistical Package for Social Sciences (SPSS) version 14 for Windows. Data were also assessed for the assumption of normality. Change in plasma data over time between treatment groups was analyzed by repeated measures analysis using the MIXED Model procedure in the SAS program. Unpaired two-tailed student's t-test was used to test the difference between treatments at post-intervention (preoperative) for the atrium results. Values were considered to be significant if $P < 0.05$.

RESULTS

No side effects of fish oil supplementation were reported except one patient complaining of fishy eructation. Two of nine patients in the placebo and two of eight patients in the fish oil group experienced atrial fibrillations following CABG surgery (Table 1). Plasma cholesterol and LDL and HDL cholesterol contents were similar in both the placebo and the fish oil–supplemented groups at baseline as well as post-intervention. Plasma triglyceride levels were significantly reduced following fish oil supplementation for 6 wk, whereas the placebo supplementation made no difference (Table 3).

The FA composition of plasma lipids was similar in the placebo and the supplement groups at baseline. LCn-3PUFA (20:5n-3, 22:5n-3, and 22:6n-3) were all significantly higher in the plasma of volunteers supplemented with fish oil concentrate. There was no change in any of the saturated, monounsaturated, or n-6 PUFA in the fish oil–supplemented compared with the placebo group (Table 4). The changes in LCn-3PUFA, calculated by subtracting the baseline FA concentrations from post-intervention values, were significantly higher in the fish oil (20:5n-3, 22:5n-3, and 22:6n-3 being 55.5 ± 29.6, 10.4 ± 7.5, and 43.8 ± 23.76, respectively) compared with the placebo group (20:5n-3, 22:5n-3, and 22:6n-3 being 1.19 ± 9.19, 1.85 ± 3.51, and 1.82 ± 11.27, respectively). Fish oil supplementation for 6 wk resulted in a significantly higher incorporation of 20:5n-3 into the lipids of atrium biopsy samples (Table 5). In the phospholipid fraction of the atrium tissue, LCn-3PUFA (20:5n-3 and 22:6n-3) were elevated following 6 wk of fish oil supplementation. This increase in LCn-3PUFA was accompanied

TABLE 3
Effects of Fish Oil or Placebo Supplementation on Blood Lipids (mmol/L)

	Placebo		Fish oil	
	Baseline	Preoperative	Baseline	Preoperative
Total cholesterol	4.81 ± 1.10	4.41± 0.58	4.86 ± 0.53	4.57 ± 0.43
HDL cholesterol	0.92 ± 0.16	0.88 ± 0.14	0.80 ± 0.14	0.73 ± 0.15
LDL cholesterol	2.81 ± 0.0.71	2.78 ± 0.37	3.40 ± 0.06	3.11 ± 0.38
Triglycerides	2.72 ± 2.64[a]	1.66 ± 1.28[a]	2.83 ± 1.52[a]	1.49 ± 0.64[b]

Values are the means ± SD. Values without a common superscript are significantly different, $P < 0.05$.

TABLE 4
FA Composition (μg/mL) of Plasma Total Lipids of Patients Prior to and Following Supplementation with Fish Oil or Placebo Capsules

	Placebo ($n = 9$)		Fish oil ($n = 8$)	
FA	Baseline	Preoperative	Baseline	Preoperative
14:0	11 ± 8	9 ± 5	14 ± 12	11 ± 9
16:0	182 ± 114	222 ± 89	238 ± 160	249 ± 141
16:1n-7	30 ± 33	24 ± 11	32 ± 25	37 ± 25
18:0	55 ± 33	73 ± 30	64 ± 40	69 ± 33
18:1n-9	213 ± 156	243 ± 112	345 ± 382	286 ± 140
18:1n-7	24 ± 10	26 ± 11	25 ± 13	30 ± 9
18:2n-6	287 ± 296	241 ± 104	188 ± 87	241 ± 107
18:3n-6	9 ± 6	9 ± 6	8 ± 6	6 ± 3
18:3n-3	7 ± 5	6 ± 3	4 ± 2	8 ± 8
20:3n-6	14 ± 8	16 ± 8	13 ± 10	12 ± 5
20:4n-6	92 ± 70	103 ± 51	91 ± 62	107 ± 56
20:5n-3	8 ± 6[a]	12 ± 6[a]	8 ± 6[a]	57 ± 29[b]
22:0	8 ± 8	7 ± 6	8 ± 8	11 ± 6
22:5n-3	6 ± 3[a]	8 ± 3[a]	5 ± 3[a]	14 ± 6[b]
22:6n-3	16 ± 8 [a]	20 ± 7[a]	13 ± 7[a]	51 ± 22[b]
Total SFA	329 ± 219	345 ± 185	260 ± 164	329 ± 133
Total MUFA	413 ± 413	364 ± 175	273 ± 190	305 ± 134
Total n-6 PUFA	402 ± 312	368 ± 119	399 ± 157	365 ± 138
Total n-3 PUFA	37 ± 21[a]	46 ± 14[a]	30 ± 19[a]	130 ± 61[b]

Values are the means ± SD. Values without a common superscript are significantly different, $P < 0.01$.

TABLE 5
FA Composition of Total Lipids Extracted from Human Atrium Tissue Collected During the CABG Surgery Following Supplementation with Fish Oil or Placebo

FA	Placebo (n = 9)	Fish oil (n = 8)
14:0	1.37 ± 0.51	1.44 ± 0.21
16:0	19.08 ± 2.84	19.23 ± 2.77
16:1n-7	3.26 ± 0.83	3.32 ± 2.22
18:0	9.02 ± 2.08	8.20 ± 1.98
18:1n-9	22.00 ± 6.01	23.28 ± 6.09
18:1n-7	2.43 ± 0.44	2.54 ± 0.36
18:2n-6	11.92 ± 1.99	13.81 ± 0.49
18:3n-3	0.37 ± 0.05	0.48 ± 0.16
20:0	0.36 ± 0.33	0.20 ± 0.19
20:1n-9	0.24 ± 0.08	0.28 ± 0.09
20:3n-6	0.53 ± 0.07	0.45 ± 0.12
20:4n-6	10.27 ± 5.28	8.44 ± 3.46
20:5n-3	0.40 ± 0.09[a]	1.35 ± 0.53[b]
22:0	0.24 ± 0.11	0.21 ± 0.06
22:5n-3	1.03 ± 0.16	0.95 ± 0.18
22:6n-3	3.05 ± 1.07	3.43 ± 1.81
Total SFA	29.91 ± 2.13	29.9 ± 1.32
Total MUFA	30.66 ± 7.15	28.13 ± 7.88
Total n-6 PUFA	22.75 ± 3.89	24.05 ± 5.26
Total n-3 PUFA	4.86 ± 1.15	6.22 ± 2.30

Values are the mean (%, wt/wt) ± SD. Values without a common superscript are significantly different, $P < 0.05$.

TABLE 6
FA Composition of Phospholipids Extracted from Human Atrium Tissue Collected During the CABG Surgery Following Supplementation with Fish Oil or Placebo

FA	Placebo (n = 9)	Fish oil (n = 8)
16:0	16.76 ± 0.66	16.30 ± 0.88
16:1n-7	0.62 ± 0.07	0.60 ± 0.2
18:0	16.60 ± .80	17.11 ± 1.94
18:1n-9	10.58 ± 0.90	10.40 ± 1.04
18:1n-7	2.40 ± 0.24[a]	2.13 ± 0.14[b]
18:2n-6	16.93 ± 0.85	17.21 ± 1.84
18:3n-6	0.00 ± 0.00	0.05 ± 0.11
18:3n-3	0.14 ± 0.12	0.21 ± 0.10
20:0	0.45 ± 0.05	0.46 ± 0.13
20:1n-9	0.10 ± 0.08	0.06 ± 0.06
20:3n-6	1.00 ± 0.19	0.89 ± 0.16
20:4n-6	21.96 ± 1.81[a]	18.49 ± 1.79[b]
20:5n-3	0.77 ± 0.28[a]	3.03 ± 0.79[b]
22:5n-3	2.07 ± 0.15	2.08 ± 0.11
22:6n-3	6.73 ± 1.21[a]	8.43 ± 1.04[b]
Total SFA	35.23 ± 0.66	35.15 ± 2.31
Total MUFA	14.92 ± 1.17	14.27 ± 1.30
Total n-6 PUFA	40.08 ± 1.34[a]	36.77 ± 1.96[b]
Total n-3 PUFA	9.76 ± 1.24[a]	13.81 ± 1.30[b]

Values are the mean (%, wt/wt) ± SD. Values without a common superscript are significantly different, $P < 0.05$.

by a reduction in 20:4n-6 content of atrium phospholipid fraction (Table 6). In the non-esterified FA fraction, no differences were found in the LCn-3PUFA (20:5n-5, 22:5n-3, and 22:6n-3) content (Table 7). FA composition in the placebo and the triglyceride fraction was similar with the exception that 20:5n-3 content was significantly elevated in the fish oil–supplemented group (Table 8).

DISCUSSION

The main purpose of this study was to examine the incorporation of LCn-3PUFA into atrium tissue lipids of patients undergoing CABG surgery. Several studies in the literature have reported insertion of LCn-3PUFA into heart membranes and their subsequent effects on heart functions, including vulnerability of the heart to becoming arrhythmic, using animal models.

TABLE 7
FA Composition of Non-esterified FA Fraction Extracted from Human Atrium Tissue Collected During the CABG Surgery Following Supplementation with Fish Oil or Placebo

FA	Placebo (n = 9)	Fish oil (n = 8)
14:0	2.29 ± 0.44	2.00 ± 0.40
16:0	15.95 ± 5.73	15.31 ± 7.67
16:1n-7	2.88 ± 2.80	2.67 ± 2.61
18:0	35.26 ± 17.88	30.02 ± 19.63
18:1n-9	17.08 ± 12.69	13.32 ± 9.76
18:1n-7	1.64 ± 0.95	7.75 ± 16.06
18:2n-6	6.41 ± 4.02	7.00 ± 2.64
18:3n-6	0.04 ± 0.07	0.20 ± 0.44
18:3n-3	0.68 ± 0.20	0.83 ± 0.53
20:0	3.33 ± 2.18	3.16 ± 2.94
20:1n-9	0.51 ± 0.42	0.54 ± 0.37
20:3n-6	4.25 ± 2.28	3.26 ± 2.35
20:4n-6	0.84 ± 0.83	1.78 ± 1.82
20:5n-3	0.69 ± 0.54	0.64 ± 0.53
22:5n-3	0.14 ± 0.24	0.70 ± 0.90
22:6n-3	0.67 ± 0.78	0.87 ± 0.61
Total SFA	65.36 ± 16.68	53.92 ± 17.35
Total MUFA	20.45 ± 13.74	26.93 ± 16.06
Total n-6 PUFA	11.91 ± 3.40	12.81 ± 3.76
Total n-3 PUFA	2.28 ± 1.35	6.34 ± 6.45

Values are the mean (%, wt/wt) ± SD.

TABLE 8
FA Composition of Triglyceride Fraction Extracted from Human Atrium Tissue Collected During the CABG Surgery Following Supplementation with Fish Oil or Placebo

FA	Placebo (n = 9)	Fish oil (n = 8)
14:0	2.21 ± 0.72	2.36 ± 0.47
16:0	18.57 ± 7.95	21.06 ± 4.17
16:1n-7	4.50 ± 3.66	4.86 ± 2.62
18:0	19.66 ± 16.58	19.00 ± 19.02
18:1n-9	26.95 ± 15.17	32.43 ± 10.19
18:1n-7	2.36 ± 1.54	2.68 ± 1.42
18:2n-6	7.19 ± 3.60	10.28 ± 3.27
20:0	1.18 ± 1.01	1.05 ± 1.20
20:1n-9	0.39 ± 0.34	0.47 ± 0.32
20:3n-6	2.52 ± 2.19	2.36 ± 2.42
20:4n-6	0.67 ± 0.65	0.67 ± 0.55
20:5n-3	0.02 ± 0.06[a]	0.16 ± 0.13[b]
22:5n-3	0.20 ± 0.23	0.46 ± 0.39
22:6n-3	0.30 ± 0.37	0.50 ± 0.51
Total SFA	43.24 ± 16.63	44.08 ± 16.77
Total MUFA	34.39 ± 20.71	40.71 ± 14.54
Total n-6 PUFA	10.52 ± 4.18	13.58 ± 2.05
Total n-3 PUFA	0.85 ± 0.95	1.64 ± 0.97

Values are the mean (%, wt/wt) ± SD. Values without a common superscript are significantly different, $P < 0.05$.

However, this is the first study to demonstrate the incorporation of dietary LCn-3PUFA into the human atrium. It is clearly evident from the results presented that 6-wk supplementation with 4.4 g/d of LCn-3PUFA results in significant enrichment of plasma and atrium lipids with these FA. It is a fair assumption to make that FA composition of atrium is reflective of myocardium tissue composition.

The phospholipid fraction of atrium lipids was particularly enriched with the major LCn-3PUFA such as 20:5n-3, 22:5n-3, and 22:6n-3. Incorporation of these FA into phospholipids is known to alter the physico-chemical property of the heart membrane and alter associated functions (21). The concomitant reduction in 20:4n-6 in the atrium phospholipids following fish oil supplementation is reflective of the preferential incorporation of LCn-3PUFA into heart membranes at the cost of n-6PUFA (22). It has been previously demonstrated that LCn-3PUFA inhibit desaturation and chain elongation of 18:2n-6 and inhibit synthesis of 20:4n-6 FA (23,24). A reduction in 20:4n-6 in the atrium tissue may result in the reduced synthesis of eicosanoids (thromboxane, prostaglandin, and leukotriene), which may further influence the heart functions as a result of fish oil supplementation (25).

This study established that 22:6n-3 is the most prevalent LCn-3PUFA in the human atrium and that the prevalence of 22:6n-3 is 7.5 times higher than that of 20:5n-3 in the atrium total lipids as well as in the phospholipid fraction of the placebo group. This is consistent with the FA data (22:6n-3 is greater than 7.5 times that of 20:5n-3) reported by Harris *et al.* (26). Sexton *et al.* (27) reported a 22:6n-3/20:5n-3 ratio of 6.0 in male and 8.5 in female human myocardium. It is evident from the results presented that the plasma FA composition is reflective of the 22:6n-3/20:5n-3 ratio (0.8) of the fish oil supplement. However, in the atrium tissue, more of 22:6n-3 than 20:5n-3 is incorporated in the atrium total lipids (22:6n-3/20:5n-3 ratio of 2.54) and phospholipids (22:6n-3/20:5n-3 of 2.78) despite fish oil supplements being higher in 20:5n-3 than 22:6n-3 content. Furthermore, it is apparent that LCn-3PUFA are enriched in phospholipids to at least twofold in comparison with those in atrial total lipids in both the placebo and the fish oil supplemented groups. Because there is only limited conversion of 20:5n-3 to 22:6n-3 in humans (28), it may be preferable to recommend 22:6n-3 enriched supplements in order to modulate cardiac membrane FA composition and, therefore, cardiac functions.

Several studies using animal models have demonstrated that dietary LCn-3PUFA prevents cardiac arrhythmias. The fibrillation threshold in animal models is significantly elevated following the feeding of a diet rich in LCn-3PUFA. It has been proposed that once the heart membranes are enriched with LCn-3PUFA, these will be preferentially released as non-esterified FA to inhibit arrhythmic events. A recent study has reported for the first time data, albeit preliminary, on acute antiarrhythmic effects of marine LCn-3PUFA in patients with implanted cardioverter defibrillators. Ventricular tachycardia was not induced in five of seven patients infused with LCn-3PUFA (29). Enrichment of the phospholipid fraction with LCn-3PUFA suggests that these FA are now readily available to be released as non-esterified acids should the arrhythmic events occur.

The incidence of atrium fibrillation was similar in the placebo and the fish oil–supplemented (2/9 and 2/8 respectively) group of patients following CABG surgery (data not shown). These results are in contrast with those of a recently published randomized controlled trial involving 160 patients that reported a 54.4% reduction in atrium fibrillations in patients supplemented with n-3PUFA for at least 5 d prior to CABG procedure compared with the control subjects (7). The sample size in the present study was too low to pick up any significant effects of fish oil supplementation on arrhythmic events. Indeed the dose of LCn-3PUFA supplemented in this study was nearly 2.5 times that used in the study by Calo *et al.* (7) and nearly 5 times that used in the GISSI Prevenzione study (8) that established prevention of ventricular fibrillation and sudden death post–myocardial infarction. Further long-term studies relating dose level, incorporation into cardiac membranes, and cardiac events are warranted to confirm the anti-arrhythmic effects of fish oils. Plasma lipid levels were similar prior to surgery in comparison with the baseline values. Although statistically insignificant, cholesterol levels tended to be lower at baseline compared with those prior to the surgery in both the placebo and the fish oil groups. It would appear that the plasma lipid lowering in a majority of the patients just prior to the surgery may be due to a combination of factors including better compliance with lipid-lowering drug (statin) therapy, better adherence to a lipid-lowering diet, and mental and physical preparation for the surgery. The fish oil–supplemented group exhibited significantly lower levels of triglyceride, which is consistent with the well-known triglyceride-lowering effects of marine LCn-3PUFA.

In summary, the results presented suggest that dietary supplementation with marine LCn-3PUFA for 6 wk increase LCn-3PUFA content of plasma and atrium lipids in humans. The timeframe for achieving the heart health benefits of LCn-3PUFA in humans remains to be investigated.

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Chlorinated Fatty Acids in Lipid Class Fractions from Cardiac and Skeletal Muscle of Chinook Salmon

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ABSTRACT: Analysis of chlorinated fatty acids (CFA) in tissues can be difficult because of their low concentrations. This task becomes even more difficult when samples are from organisms living in remote locations with very little exposure to environmental contamination. Therefore, enrichment of CFA is necessary prior to analysis. In this study, CFA were enriched from fractionated lipid classes of cardiac and skeletal muscle of Chinook salmon (*Oncorhynchus tshawytscha*) to determine CFA distribution among lipid classes and tissue types and to demonstrate the sensitivity of this method to detect CFA at trace concentrations. The lipids extracted from cardiac and skeletal muscle of *O. tshawytscha* were separated into fractions containing TAG, FFA, and phospholipids. After transesterification, the FAME derivatives from each lipid class were analyzed by GC with a halogen-selective detector (XSD) to determine the concentrations of dichlorostearic acid and dichloropalmitic acid. Other chlorinated compounds detected by GC-XSD were analyzed by GC–MS. CFA were observed in all lipid classes in both cardiac and skeletal muscle tissues. However, the highest concentrations of CFA were found in the phospholipids of both tissue types, about 1–2 mg/g lipid. It was also shown that dichloropalmitic acid concentrated in cardiac phospholipids whereas dichlorostearic acid was found primarily in the phospholipids of skeletal tissue. CFA concentrations in TAG and FFA fractions were below 150 mg/g lipid. This study demonstrates a small-scale approach to the study of CFA at trace concentrations and their distribution among lipid classes.

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Chlorinated fatty acids (CFA) are found in almost all marine biota. However, the concentrations vary greatly depending on the environmental load of chlorine to which they are exposed. The increased chlorine load on the environment from anthropogenic sources such as effluents from pulp bleach mills and flour bleaching processes has lead to increased concentrations of extractable chlorinated organic compounds present in marine organisms (1). Chlorinated compounds such as PCBs, DDT, dioxins, and other persistent organic pollutants are widespread in the environment and have received considerable attention owing to their bioaccumulation in marine biota and their resulting physiological effects (2–6). However, these compounds constitute less than 5% of the total extractable chlorinated organic compounds (7–10). The majority of the remaining chlorine resides in chlorinated fatty acids (CFA) (7,8,11). The potential physiological impacts of CFA are not fully understood, but research has shown that high CFA concentrations affect reproduction and sperm motility, cause abnormal weight gain of certain organs of rats, and cause cell membrane disturbances (12–14).

The uptake and turnover of CFA are similar to those of other FA (15). CFA are incorporated into phospholipids and into cell membranes. The resulting effects are changes in membrane fluidity, interference with ion transport and membrane-bound enzymes, and ATP leakage through mitochondrial membranes brought about by the conformational change to the FA owing to the large radii of chlorine (12,13). Despite these physiological effects, CFA are not recognized by the tissues as xenobiotic and are not eliminated from the cell. Studies on the influence of CFA on the activation of cytochrome P450, the enzyme responsible for the removal of xenobiotics and toxic substances, have shown that CFA do not induce enzymatic detoxification in response to these compounds (12).

The limitation in the study of CFA is their relatively low concentrations compared with that of other FA. To compensate, enrichment of CFA must be performed before analysis of the samples can be successful. However, even with the enrichment, large sample volumes are needed to detect CFA at trace levels such as those found in marine organisms from remote areas. Improvements in enrichment methods allow for use of smaller sample volumes and therefore open new research opportunities by making available new sample sources.

This study is aimed at demonstrating the sensitivity to detect trace CFA concentrations, and at determining CFA distribution among lipid classes and tissue types using a solid-phase extraction (SPE)-based enrichment method. In this method, the removal of saturated FA is accomplished using SPE fractionation techniques as opposed to a urea complexation step most commonly used in CFA enrichment. The sufficient removal of saturated FA by SPE will eliminate the need for the use of urea, which is time consuming and can produce low recoveries of CFA. The expected outcome is a higher CFA concentration per total lipid in the final enriched fraction. This will allow for analysis of the CFA by halogen-sensitive detector (XSD) without losing sensitivity due to the selectivity of the instrument in the presence of nonhalogenated compounds. At higher XSD reactor temperatures, such as 1100°C used in this study, sensi-

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Abbreviations: ASE, accelerated solvent extraction; CFA, chlorinated FA; PICI, positive ion chemical ionization; PL, polar lipid; SPE, solid-phase extraction; XSD, halogen-selective detector.

tivity for chlorinated compounds is increased, but the advantage is lost without enrichment due to a decrease in selectivity of the XSD for the chlorinated compounds (16).

Chinook salmon (*Oncorhynchus tshawytscha*) tissue samples used in this study were obtained from the Kenai River, Alaska. This is an optimal tissue source for testing the sensitivity of this method because of the life cycle of *O. tshawytscha* and their resulting low levels of contaminants. Chinook salmon are an anadromous species, which spend the majority of their lives in the ocean, only returning to their natal freshwater to spawn. A tissue amount of 1 g was used from both cardiac and skeletal muscle tissues. The resulting FA from the different lipid classes applied to the enrichment procedure had masses ranging from 5 mg down to 200 mg total lipid. This study demonstrates the sensitivity of the improved methodology and the opportunity provided for the microscale analysis of CFA in muscle tissues.

MATERIALS AND METHODS

Chemicals. All reagents used in this study were of analytical grade. The following chemicals were used: chloroform (#049, >99.9%; Burdick & Jackson, Muskegon, MI), dichloromethane (#DX0837, >99.9%; EMD, Gibbstown, NJ), methanol (#VW4303, >99.8%; VWR, West Chester, PA), ethanol (#BDH1156, 89–91%; VWR), 2,2,4-trimethylpentane (#9335, >99.8%; J.T. Baker, Phillipsburg, NJ), *n*-hexane (#442437, >95%; Aldrich, Milwaukee, WI), ammonium hydroxide (#AX1303, 28.0–30.0%; EMD), sodium sulfate (#SX0760, >99.0%; EMD), potassium chloride (#P3911, 99.0–100.5%; Sigma Chemical, St. Louis, MO), silver nitrate (#2179, >99.7%; J.T. Baker), 2,6-di-*tert*-butyl-4-methylphenol (BHT; #B1378, >99.0%; Sigma), ethyl ether (#106, >99.9%; Burdick & Jackson), sodium acetate (J.T. Baker 3460, 99.0-101.0%), sulfuric acid (SX1247, 95.5–96.5%; EMD), acetic acid (#9507, >99.7%; J.T. Baker), hydromatrix drying agent (Varian, Palo Alto, CA), helium (>99.9999%; Air Liquide America, Houston, TX), hydrogen (#9200, >99.9995%; Airgas, Anchorage, AK). Glassware was washed and solvent-rinsed before use. FA and TAG standards were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). CFA standards dichloropalmitic acid and dichlorostearic acid were prepared from palmitoleic acid and oleic acid, respectively (17).

Preparation of standards. CFA standards were synthesized in an ionic liquid. The liquid was prepared by combining equimolar amounts of 1-butyl-3-methylimidazolium chloride and aluminum chloride (10 and 7.6 g, respectively) in an inert nitrogen atmosphere. Each specific unsaturated FA, such as methyl oleate (1 g), was added with stirring, and chlorine gas was supplied to the mixture for 30 min. After stirring the reaction mixture for another 30 min, the product was extracted with *n*-hexane and the solvent stripped with a stream of nitrogen (17).

Tissue samples. Cardiac and skeletal muscle tissues from *O. tshawytscha* were collected during May and June of 2005 from sport-caught fish from the Kenai River, Alaska, and from the Alaska Department of Fish & Game Kenai River King Salmon netting project. The fish were removed from the Kenai River between the Soldotna Bridge at river-mile 20 and Warren Ames Bridge at river-mile 6.0. No sources of industrial contamination influence the water quality of the river. Upon removing the fish from the water, the entire heart and skeletal muscle samples were immediately extracted and stored on ice until leaving the field, after which they were vacuum-sealed and stored at –20°C.

Sample preparation. Samples were removed to room temperature and thawed. Entire hearts and several grams of skeletal muscle from each fish were minced separately using a stainless steel knife. The mass of each sample was determined gravimetrically. The samples were then placed in a stainless steel blender and diluted 1:1 (by wt) with saline solution and further homogenized. From the tissue homogenates, 2-g samples, representing 1 g tissue before dilution, were weighed on an analytical balance (model AT201; Mettler, Columbus, OH), and the exact masses recorded. Approximately 1 g of hydromatrix drying agent (Varian) was mixed with each sample portion. The mixture was transferred to 11-mL stainless steel cells containing two cellulose filters (Dionex, Sunnyvale, CA) for extraction by accelerated solvent extraction (ASE) system (ASE 200; Dionex). Additional hydromatrix was added to fill each cell.

Lipid extraction. Lipids were extracted by ASE using a method modified by Dodds *et al.* (18). The ASE was operated at 100°C and 2000 psig (13.8 MPa) for two 5-min static extraction cycles using a chloroform/methanol (65:35, vol/vol) solvent system. Chloroform was prepared with BHT at a concentration of 100 mg/L to prevent oxidation of samples.

To sample collection vials, about 30 mL 0.7% NaCl solution was added to remove any proteins present in extract and to partition the methanol from the chloroform phase containing the extracted lipids. Vials were vortexed and allowed to separate for about 30 min until complete phase separation occurred. The aqueous layer was then removed and discarded. The remaining extract was dried by pouring through a sintered funnel containing anhydrous sodium sulfate into TurboVap (Zymark, Hopkinton, MA) evaporation vessels. Vials and funnels were thoroughly rinsed several times with chloroform to ensure quantitative transfer of lipid content.

Extracts were dried under nitrogen using a TurboVap II solvent evaporation system at a water bath temperature of 35°C. Lipid residues in each tube were immediately redissolved in *n*-hexane and quantitatively transferred to preweighed vials. Solvent was removed under a gentle stream of nitrogen, and exact masses were determined gravimetrically to 0.01 mg. Lipids were quickly dissolved in hexane/chloroform/methanol (95:3:2, by vol) for a concentration of 20 mg/150 μL for lipid class fractionation.

Lipid class fractionation. Lipids were separated into FFA, nonpolar and polar lipid fractions using a solid-phase extraction (SPE) procedure modified from Ruiz *et al*. (19). A Visiprep vacuum manifold (Supelco, Bellefonte, PA) was set up with 500 mg aminopropyl SPE minicolumns (Supelclean LC-NH_2 SPE Tubes, 3 mL, 50 mg loading capacity; Supelco,

No. 57014). SPE columns were conditioned with 5 mL *n*-hexane at a flow rate of 1–2 mL/min. Flow was stopped when the solvent level reached the solid phase. The column was loaded with 150 mL lipid extract containing 20 mg total lipid. The flow rate was adjusted to 0.5–1 mL/min, and fractions were collected in separate vials. Fraction 1 (TAG), containing nonpolar TAG, was eluted with 5 mL chloroform. Fraction 2, containing FFA, was eluted with 5 mL diethyl ether/acetic acid (98:2, by vol). The polar lipids of fraction 3 (PL) were eluted with 2.5 mL methanol/chloroform (6:1, vol/vol) followed by 2.5 mL 0.05 M sodium acetate in methanol/chloroform (6:1, vol/vol). FFA and PL were washed with 3 mL H_2O to remove acetic acid from FFA and to remove sodium acetate and methanol from PL. The organic phases were transferred to new vials and dried over anhydrous sodium sulfate. Fractions were transferred to round-bottomed reaction tubes and evaporated to dryness under a gentle stream of nitrogen.

Transmethylation. Each lipid fraction was subjected to an acid-catalyzed transesterification to produce FAME derivatives (20). All recovered lipids from each fraction were dissolved in 2 mL 1% sulfuric acid (by vol) in methanol. Tubes were placed on heating blocks at 70°C overnight (at least 12 h). Samples were removed from heat and cooled to room temperature. To each, 1 mL hexane and 2 mL H_2O were added. Tubes were vortexed and centrifuged at 1315 × *g* (BD-Clay Adams 0131, Franklin Lakes, NJ) for 3 min.

The hexane phase was removed to a new vial. An additional 1 mL of hexane was added to the aqueous phase in each reaction tube and then vortexed and centrifuged as before. The hexane phase was removed, and the two hexane phases were combined and dried over anhydrous sodium sulfate. Dried solutions were decanted to new vials and evaporated under a gentle stream of nitrogen. Masses of remaining FAME were determined gravimetrically. FAME were immediately dissolved in hexane for 200 µL SPE sample loading at concentrations listed in Table 1.

Chlorinated FAME isolation by SPE. CFA were isolated from saturated and monounsaturated FAME using an SPE method modified from Åkesson-Nilsson *et al*. (21). The vacuum manifold and LC-NH_2 SPE column were used as described previously. After the columns had been conditioned with hexane, 200 µL of the FAME solution was applied. In this separation, two fractions were collected. The first fraction (f1), which contains primarily saturated and monounsaturated FAME, was eluted with *n*-hexane. The second fraction (f2), containing CFA, was eluted with hexane/diethyl ether/dichloromethane (89:1:10, by vol). FAME from f2 were evaporated to dryness under nitrogen, then dissolved in 1 mL 2,2,4-trimethylpentane. The f1 fractions were evaporated under nitrogen and redissolved in 1mL *n*-hexane for GC analysis. Excess FAME from transesterifaction not used in SPE separation were prepared in *n*-hexane for GC analysis of total lipid content.

Removal of polyunsaturated FAME. Enrichment of CFA by the removal of polyunsaturated FAME was performed using a method modified from Mu *et al*. (22). To each f2 sample in 2,2,4-trimethylpentane, approximately 1 mL 1.5 M $AgNO_3$ in ethanol/H_2O (1:1, vol/vol) was added and mixed thoroughly to remove PUFA. The phase containing uncomplexed FAME and CFA was decanted to a new vial. The $AgNO_3$ phase was washed with an additional 1 mL 2,2,4-trimethylpentane, which was removed and combined with the first extraction and dried over anhydrous sodium sulfate. To the $AgNO_3$ phase, 1 mL H_2O was added and polyunsaturated FAME were extracted with 1 mL *n*-hexane three times. The hexane phases were combined and dried over sodium sulfate. All samples were transferred to new vials and evaporated under nitrogen. They were then redissolved in *n*-hexane for GC analysis.

TABLE 1
FAME Recovered from Each Lipid Class[a] and Applied to SPE Isolation of CFA[b]

Lipid fraction-tissue type[c]	FAME (mg)	FAME applied to SPE (mg)
TAG-C	3.67–5.92	3
TAG-S	11.11–13.07	5
FFA-C	5.31–6.52	5
FFA-S	2.16–3.54	2
FFA-S (sample 6)[d]	2.02	1
PL-C (sample 1)[d]	0.96	0.75
PL-C	1.12–1.68	1
PL-S	0.25–1.19	0.200

[a]Quantity of FAME received from each lipid class separated from 20 mg total lipid.
[b]Amount of FAME per 200 µL of *n*-hexane applied to solid-phase extraction (SPE) of chlorinated fatty acids (CFA).
[c]C, cardiac muscle; S, skeletal muscle; PL, polar lipid.
[d]Amount of FAME applied to SPE for these samples varies from other samples of same lipid class and tissue type.

GC. The FAME were analyzed for dichloropalmitic and dichlorostearic acids and other chlorinated compounds using a Varian CP-3800 gas chromatograph equipped with an XSD detector (#5360; OI Analytical, College Station, TX). The samples were injected using a splitless injection for 1 min onto a fused-silica capillary column (DB23, 60 m × 0.25 mm i.d.; film thickness, 0.25 mm; J&W Scientific, Folsom, CA). Helium was used as a carrier gas at a flow rate of 1 mL/min with a pressure pulse of 25 psi for 0.5 min. The injector temperature was set at 300°C. The column temperature was raised from 125 to 250°C at 3°C/min and held at 250°C for 20 min. The XSD reactor temperature was set at 1100°C. Purified air was used as the reaction gas at a flow of 12–20 mL/min.

MS. GC–MS was used to calculate recoveries and to identify unknown compounds. The Varian GC and DB23 column were connected to a Varian Saturn 2200 ion trap mass spectrometer, and the same GC conditions and temperature program as above were used for GC–MS analysis. For electron impact ionization (EI), the electron energy was 70eV with a source temperature of 120°C. Ammonia was used as the reagent gas for positive-ion chemical ionization (PICI).

RESULTS AND DISCUSSION

The XSD chromatograms indicated that halogenated compounds were present in all lipid classes of both types of muscle tissue (Table 2). Dichloropalmitic acid ($C_{16}Cl_2$) was found in high concentrations in both skeletal and cardiac muscle tissue,

TABLE 2
CFA Concentrations[a] Following Enrichment in All Lipid Classes of Cardiac and Skeletal Muscle from *Onchorhynchus tshawytscha*

	Total CFA (μg/g lipid)	$C_{16}Cl_2$ (μg/g)	$C_{18}Cl_2$ (μg/g)	Unknown (μg/g)
Cardiac muscle				
Phospholipids	1187	1098	0	88.7
FFA	144	35.1	15.8	93.4
TAG	55.4	8.4	10.4	36.6
Skeletal muscle				
Phospholipids	2197	855	634	708
FFA	15.2	0	0	15.2
TAG	131	0	0	131

[a]Concentration were quantified by GC–halogen-selective detector (XSD) using dichloropalmitic ($C_{16}Cl_2$) and dichlorostearic ($C_{18}Cl_2$) acid methyl ester external standards. Unknown CFA were calculated using the calibration for dichloropalmitic acid methyl ester. For abbreviation see Table 1.

whereas dichlorostearic acid ($C_{18}Cl_2$) was found mainly in skeletal muscle phospholipids (Fig. 1). Other chlorinated compounds were found in all fractions; however, the most diverse group was found in the nonpolar TAG fractions (Fig. 2). The predominant unknown chlorinated compound was tentatively identified as dichloromyristic acid ($C_{14}Cl_2$) based on retention time and GC–MS analysis (Fig. 3). The retention time of dichloromyristic acid was calculated by extrapolation from the plot of $C_{16}Cl_2$ and $C_{18}Cl_2$ retention times given the linear elution of homologs.

The intensity of *m/z* of 69, 74, and 87, are consistent with the mass spectrum of myristic acid methyl ester. 5,6-Dichloromyristic acid has been previously identified as a major constituent in marine biota (11). Other peaks, which could not be identified by GC–MS owing to low concentrations and coelution with other FAME, were possibly chlorohydroxy FA or chlorinated sulfones. These compounds also have been previously identified in some marine biota (23). Statistical analysis by one-way ANOVA ($P = 0.05$) showed no significant difference between CFA concentrations in cardiac and skeletal muscle, or between CFA concentrations in corresponding lipid classes from these tissues. However, there are significant differences in CFA species between the two compounds. $C_{16}Cl_2$ was found at higher concentrations in cardiac tissue, whereas $C_{18}Cl_2$ was found at detectable levels only in skeletal muscle. This differential uptake and incorporation of CFA species may be correlated with the aforementioned physiological effects attributed to CFA.

In the phospholipid fractions of both tissue types, co-elution of two isomers of dichlorostearic and dichloropalmitic acids was evident on the chromatograms (Fig. 4). The second of the two peaks to elute is possibly a dimethyl acetal derivative resulting from the transmethylation of the vinyl-ether bound FA present in phospholipids (24). Because of the resolution and similar mass spectra of FAME and FA dimethyl acetals, the identification of these compounds could not be confirmed. In the cardiac muscle phospholipids, roughly 20–35% of the $C_{16}Cl_2$ was in the suspected dimethyl acetal conformation. A very small amount of dichlorostearic dimethyl acetal was present in any fraction. To confirm identification of these compounds in future experiments, the phospholipid fractions could be further separated into constituent molecular species using an SPE method prior to transmethylation and CFA enrichment (25). This could result in the separation of the methyl ester and dimethyl acetal derivatives since ether-bound FA are primarily found in ethanolamine phospholipids (26). However, this separation of phospholipid species would require a larger sample size than used in this study owing to the small PL content of the samples. This separation may also result in the inability to detect small CFA concentrations otherwise detectable in a total phospholipid fraction.

The extraction by ASE yielded about 30 mg lipid per g of cardiac tissue, and an average of about 60 mg lipid per g skeletal muscle. This coincides with previous findings of roughly

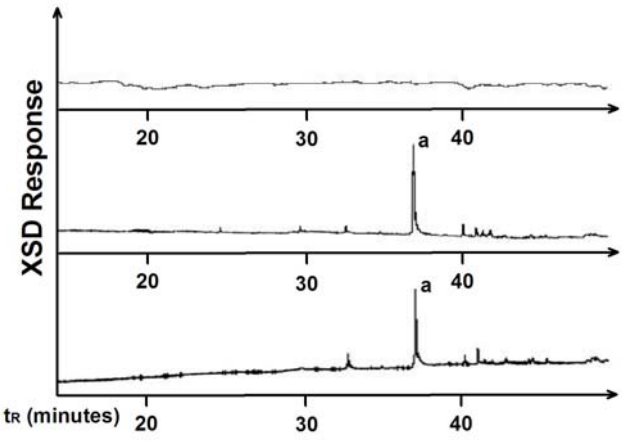

FIG. 1. Halogen-selective detector (XSD) chromatograms of samples before chlorinated FA (CFA) enrichment and after enrichment. Samples were from phospholipid fractions of cardiac and skeletal muscle of chinook salmon. Top chromatogram is of a sample containing FAME from cardiac phospholipids before the enrichment procedure. The sample was run at a concentration of 1 mg total lipid/mL. At this concentration, CFA were not at detectable levels. Middle and bottom chromatograms are after CFA enrichment. The middle is from cardiac muscle and the bottom from skeletal muscle. (a) Dichloropalmitic acid methyl ester.

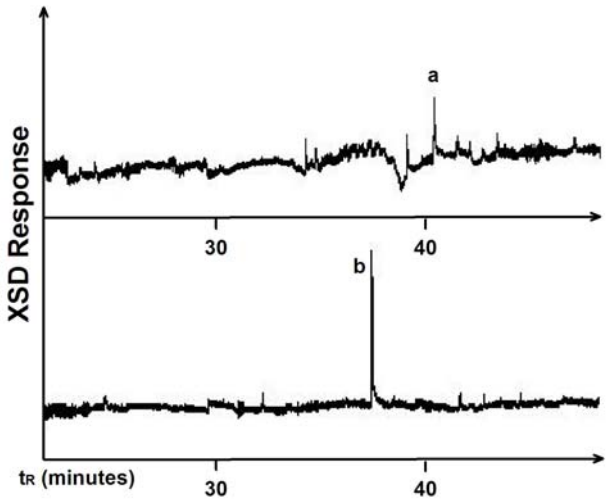

FIG. 2. XSD chromatograms of CFA enriched from TAG fractions of cardiac and skeletal muscle. Top chromatogram is from cardiac muscle, bottom is from skeletal muscle. (a) Dichlorostearic acid methyl ester, (b) dichloropalmitic acid methyl ester. For abbreviations see Figure 1.

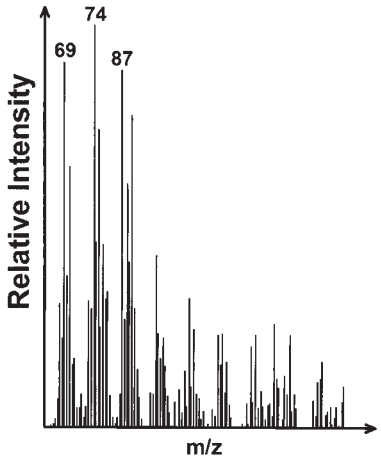

FIG. 3. EI mass spectrum of dichloromyristic acid methyl ester from skeletal muscle TAG. Peaks at *m/z* of 69, 74, and 87 are characteristic of a saturated FA.

6–7% lipid content in skeletal muscle of *O. tshawytscha*. The percent recovery of total FA for the extraction and transesterification was determined using a trinonadecanoic acid (19:0) surrogate standard, which was spiked in the tissue samples at a concentration of 50 μg/mL prior to lipid extraction. After extraction, lipid class fractionation, and transesterification, 19:0 was measured and a recovery was calculated of 76% (±2.9%). This surrogate recovery is within the expected range given the previously reported recoveries of the individual methods used. The losses can be attributed to the combination of an 80–90% recovery of lipids by ASE, high recoveries by SPE lipid fractionation, and the additional loss of phosphoserine, phosphocholine, phosphoethanolamine, and other phospholipid derivatives during the transesterification process, which constitute about one-third of the mass of phospholipids (27).

To measure percent recoveries of FAME for the enrichment of CFA, quantities of the following were measured: myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9), EPA (20:5n-3), and DHA (22:6n-3). These compounds were chosen because previous studies found them to be the primary FA in salmon (Fig. 5). This combination of saturated, monounsaturated, and polyunsaturated FAME is sufficient to demonstrate the effectiveness of the CFA enrichment method. SPE isolation of CFA was used to remove 14:0, 16:0, 18:0, and 18:1n-9, among other trace saturated FA and short-chain unsaturated FA. Silver ion complexation was used to remove unsaturated FA, primarily 20:5n-3 and 22:6n-3, found in salmon tissue.

The recoveries of 14:0, 16:0, and 18:0 from the SPE enrichment were within 20% of the initial quantities of lipid applied to the SPE column for the FFA and the cardiac TAG fractions (Table 3). The recovery of 18:1n-9 was lower in these fractions, between 64 and 85%. Recoveries for these FAME in skeletal muscle TAG were from 127 to 149%. These high recoveries may be attributed to incomplete elution of the surrogate in the first fraction of this SPE separation procedure. This would increase the apparent concentration of these target compounds in this fraction. The SPE method described by Åkesson-Nilsson (21) did not use 19:0 in the development of this method. The levels of FAME from the phospholipid fractions of cardiac muscle were highly variable, from 12 to 82%, whereas there was no recovery for saturated FAME in the skeletal muscle phospholipids. The reason for the variability in the cardiac phospholipids is that FAME were at low concentrations owing to the small initial lipid mass (0.75–1.00 mg total lipid) applied to the SPE column. The resulting recoveries were near detection limits for some of these compounds. Detection limits of the GC–MS method used for calculating these recoveries were between 4 and 13 μg/mL. The lack of recovery of saturated FAME in the skeletal tissue was also due to the low initial lipid

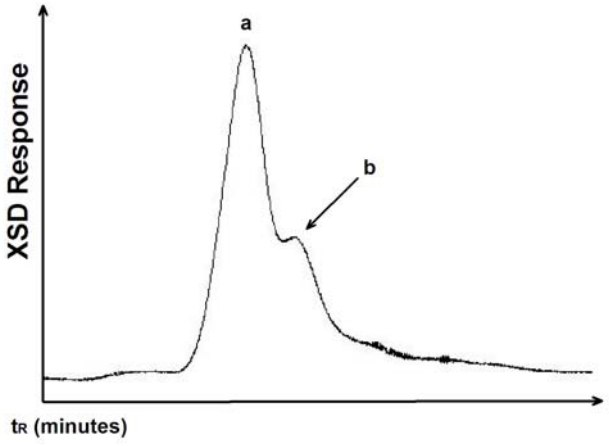

FIG. 4. XSD chromatogram of dichloropalmitic acid isomers. (a) Dichloropalmitic acid methyl ester, (b) tentatively identified dichloropalmitic dimethyl acetal. For abbreviation see Figure 1.

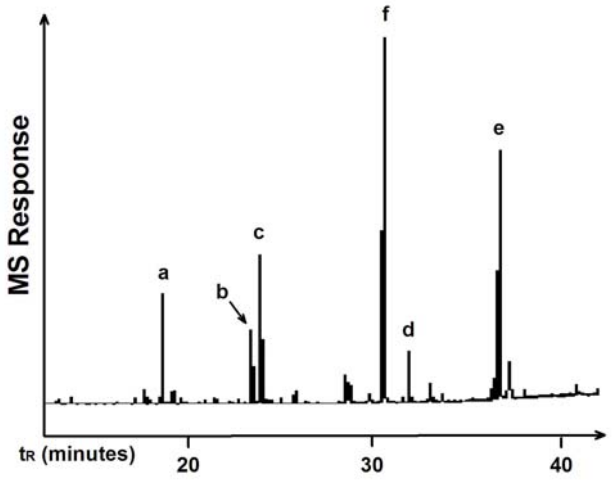

FIG. 5. Content of total FAME content liberated from unfractionated lipids of salmon muscle tissue. (a) Palmitic acid methyl ester, (b) stearic acid methyl ester, (c) oleic acid methyl ester, (d) EPA methyl ester, (e) DHA methyl ester, (f) internal standard.

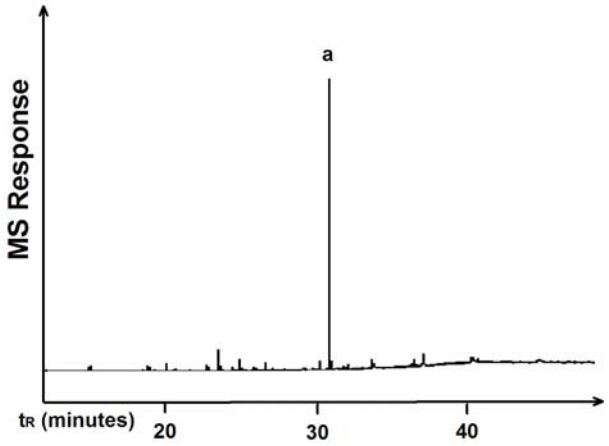

FIG. 6. GC–MS chromatogram of enriched CFA fraction from salmon cardiac FFA. (a) Internal standard. For abbreviation see Figure 1.

mass (200 μg total lipid). Any amounts of saturated FA recovered were below the limits of detection.

The recoveries of polyunsaturated FAME from the silver ion complexation were low for all lipid fractions except for 22:6n-3 in the skeletal TAG and 20:5n-3 in skeletal phospholipids. The rest showed between 39 and 77% recoveries. This was to be expected as a large portion of polyunsaturated FAME is not eluted in the two fractions during the SPE enrichment procedure (21). The unsaturated FAME have a greater dipole interaction with the NH_2 column and require a more polar solvent than the hexane/diethyl ether/dichloromethane (89:1:10, by vol) solvent mixture used to elute the CFA. The high recovery of 22:6n-3 in the skeletal TAG may be have the same cause as the high recoveries of other FAME in this lipid class. The high recovery of 20:5n-3 in the skeletal PL is likely a result of low FAME concentrations, i.e., near the limits of detection.

TABLE 3
Percent Recoveries and SD of Primary FAME in All Salmon Lipid Classes for CFA Enrichment Following Transesterification[a]

	Cardiac muscle		Skeletal muscle	
	% Recovery[b]	SD	% Recovery[b]	SD
TAG FAME				
14:0	79.3	17.8	144.8	21.2
16:0	83.3	12.6	149.0	22.7
18:0	82.0	12.7	138.2	19.5
18:1n-9	64.4	38.0	127.9	17.6
20:5n-3	62.0	27.3	39.1	11.5
22:6n-3	53.7	11.8	112.8	35.9
FFA FAME				
14:0	119.9	24.1	112.9	17.3
16:0	106.8	20.6	101.5	15.9
18:0	111.0	21.9	102.8	9.2
18:1n-9	84.7	18.3	107.1	35.5
20:5n-3	66.2	14.1	77.1	22.3
22:6n-3	57.5	5.5	75.5	30.0
Phospholipid FAME				
14:0	11.7	8.9	—	—
16:0	69.8	35.3	—	—
18:0	82.3	38.3	—	—
18:1n-9	60.9	38.6	43.3	36.1
20:5n-3	46.2	17.2	126.3	16.1
22:6n-3	40.2	17.3	46.6	16.6

[a]Samples were analyzed in triplicate.
[b]Recoveries for saturated and monounsaturated FA were calculated using data from SPE fractions. Polyunsaturated FA recoveries were calculated from silver ion complexation recoveries. For abbreviations see Table 1.

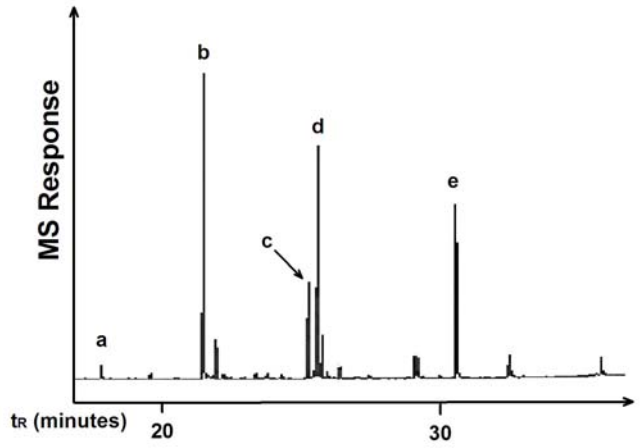

FIG. 7. Chromatogram of first fraction from solid-phase extraction enrichment of CFA in cardiac TAG. (a) Myristic acid methyl ester, (b) palmitic acid methyl ester, (c) stearic acid methyl ester, (d) oleic acid methyl ester, (e) internal standard. For abbreviation see Figure 1.

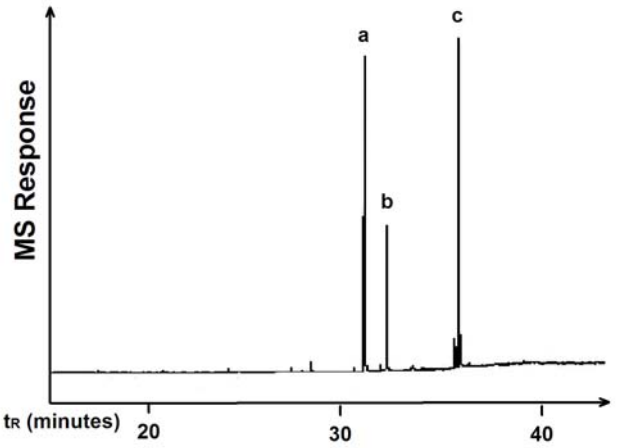

FIG. 8. GC–MS chromatogram of FAME recovered from silver ion complexation procedure on cardiac tissue TAG. (a) Internal standard, (b) EPA methyl ester, (c) DHA methyl ester.

After enrichment, GC analysis of the fractions containing the CFA did not indicate any significant quantities of nonchlorinated FAME (Fig. 6). As seen in Figure 6, the only quantifiable peak was that of the internal standard. All recoveries of nonchlorinated FAME in the enriched fractions were below detection limits for GC–MS.

None of the f1 fractions from the SPE procedure, containing primarily saturated FAME, showed significant levels of long-chain polyunsaturated FAME (Fig. 7). Any observed peaks were below limits of detection. Likewise, chromatograms from the silver ion complexation procedure did not indicate the presence of saturated or monounsaturated FAME (Fig. 8). This agrees with previous studies showing that saturated FAME will not complex with Ag, and that CFA, which act as saturated FAME in nature, also will not form Ag complexes (10).

XSD analysis of f1 fractions and the FAME recovered from the silver ion complexes showed that no CFA were present in these solutions. This demonstrates an accurate separation of CFA, saturated FAME, and unsaturated FAME by this method. The removal of the predominant saturated FAME by SPE, namely, 14:0, 16:0, and 18:0, eliminates the need for the removal of these compounds by urea complexation, a procedure that is time consuming and yields a lower recovery than the SPE method. With the removal of most of the dominant FA species present in these tissues, the final CFA fraction can be concentrated for CFA enrichment.

The method for the enrichment of CFA used in this experiment allows for the detection of CFA at trace concentrations. The combination of this CFA enrichment and XSD detection limits near 100 pg Cl allows detection of CFA in tissue samples at concentrations less than 80 ng/g. This makes this method ideal for measuring CFA levels in organisms living in non-chlorine-loaded habitats, such as *O. tshawytscha* that spend that majority of their life in the open ocean. The sensitivity of this method also allows for the use of sample sizes of less than 1 g, depending on the lipid class or compound of interest. The 1 g sample mass used in this experiment was sufficient for all CFA analyses except on the skeletal muscle PL fractions. The reason this sample size was not large enough for this fraction is that the proportion of phospholipids is small compared with that of TAG (the major fat storage molecule) in the skeletal muscle of salmon. Of the 20 mg total lipid used for the lipid class fractionation, only about 0.5 mg FAME were recovered from the PL fraction. Despite the inability to verify the method from FAME recoveries for this lipid class owing to concentrations below GC–MS detection limits, high concentrations of CFA were detected by XSD after the enrichment procedure.

The XSD chromatograms in Figure 1 demonstrate the need for enrichment of CFA for analysis at trace concentrations. The top chromatogram shows total FAME before the enrichment procedure. CFA are not detectable because of their low concentrations with respect to nonchlorinated FAME. The total FAME content before enrichment prevents samples from being concentrated down to volumes necessary for CFA detection because of the resulting overloading of sample on the GC column and the reduction of XSD selectivity for CFA. As shown in the bottom two chromatograms of Figure 1, CFA enriched through this procedure can be concentrated to adequate levels for XSD detection.

In future experiments it may be beneficial to use Ag-impregnated LC-NH_2 SPE columns, a method described by Christie (20) that eliminates the separate silver ion complexation step. This would decrease the chances of CFA loss during solution transfers between the SPE and silver ion complexation procedures.

The differential uptake and incorporation of primary CFA species between both lipid classes and tissue types are subjects that need further investigation. The specific accumulation of dichloropalmitic acid in cardiac tissue and the similar accumulation of dichlorostearic acid in skeletal muscle may be directly

linked to the development of physiological defects. Although not obvious in *O. tshawytscha* given the life cycle of this fish, the physical condition of organisms exposed to high levels of chlorine in their environment may be adversely affected. However, there has been limited research focused on the distribution of CFA among lipid classes and among different tissue types.

Thus, the sequential method of CFA enrichment described in this experiment using SPE separation techniques appears to be a sensitive method for the isolation and analysis of trace levels of CFA. This small-scale approach to the analysis of CFA in biological tissues could make it possible for the development of less-invasive sample collection procedures to be used on live organisms given the small sample size requirements. This study also demonstrates the persistence of CFA in marine biota as well as their concentration in skeletal and cardiac muscle phospholipids.

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Effects of Flaxseed Derivatives in Experimental Polycystic Kidney Disease Vary with Animal Gender

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ABSTRACT: Flaxseed derivatives, including both oil and flax lignan, modify progression of renal injury in animal models, including Han:SPRD-*cy* polycystic kidney disease (PKD). Gender is a significant factor in the rates of progression of many forms of human renal disease, but the role of gender in the response to nutrition intervention in renal disease is unexplored. In this study, male and female Han:SPRD-*cy* rats or normal littermates were fed either corn oil (CO) or flax oil (FO) diets, with or without 20 mg/kg of the diet flax lignan secoisolariciresinol dyglycoside (SDG). Renal injury was assessed morphometrically and biochemically. Renal and hepatic PUFA composition was assessed by GC and renal PGE_2 release by ELISA. FO preserved body weight in PKD males, with no effect in females. SDG reduced weight in both normal and PKD females. FO reduced proteinuria in both male and female PKD. FO reduced cystic change and renal inflammation in PKD males but reduced cystic change, fibrosis, renal inflammation, tissue lipid peroxides, and epithelial proliferation in PKD females. SDG reduced renal inflammation in all animals and lipid peroxides in PKD females. A strong interaction between SDG and FO was observed in renal FA composition of female kidneys only, suggesting increased conversion of C_{18} PUFA to C_{20} PUFA. FO reduced renal release of PGE_2 in both genders. Gender influences the effects of flaxseed derivatives in Han:SPRD-*cy* rats. Gender-based responses to environmental factors, such as dietary lipid sources and micronutrients, may contribute to gender-based differences in disease progression rates.

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Although diet as a strategy to slow the progression of renal disease has yet to make a major impact in clinical medicine, it remains a potent strategy in experimental renal disease. Despite a paucity of encouraging human results, and, indeed, a lack of clinical trials of sufficient duration and rigor to explore effects on the early phases of kidney disease, patient interest in diet and other nonpharmacologic strategies to slow renal disease continues to grow (1). The interest of the medical community in nutrition as a prevention strategy in renal disease progression, on the other hand, seems to be waning. The annual international Prevention in Renal Failure conferences organized through the University of Toronto have not included a section on nutrition for the last 5 years.

Flaxseed has been demonstrated to have possible benefits in the reduction of inflammation in rheumatic disease, reduction of atherosclerosis and modification of growth of hormone-dependent tumors (2–6). A possible mechanism for health benefits of flaxseed includes enrichment with the PUFA α-linolenic acid (ALA). Such enrichment has been associated with reduction in pro-inflammatory prostanoids or cytokines in circulating mononuclear cells (7), particularly tumor necrosis factor α and interleukin-1β (8). Flaxseed is also the richest natural source of lignans, present in the husk of the seed as the ester secoisolariciresinol diglycoside (SDG), which is hydrolyzed in the intestinal lumen to enterodiol and enterolactone (9). SDG or its hydrolyzed products may influence health or disease through estrogenic pathways, an antioxidant effect, or through antagonism of platelet activating factor (10). SDG is, however, lipophobic, and refined flax oil (FO) contains only negligible amounts of SDG, making it possible to separate the contribution of the oil and the lignan experimentally. In addition, recent studies have suggested that flaxseed meal, which is enriched in flax protein but contains little oil or lignans, ameliorates experimental diabetic nephropathy (11). We have demonstrated that partial dietary substitution of ground flaxseed, pure FO, and the addition of SDG can modify histologic markers of renal injury early in the course of disease in the male Han:SPRD-*cy* rat (12–14). This complements previous studies that showed benefits of flaxseed or FO in the rat 5/6 nephrectomy model (15) and murine or human lupus erythematosus (16,17).

The role of gender as a factor that influences progression of renal disease, particularly polycystic kidney disease (PKD), has been the subject of growing interest. Both Gabow *et al.*, in a population in which the PKD genotype was not known (18), and Magistroni *et al.* in a cohort of patients identified at PKD2 demonstrated longer renal survival in female patients (19), although Yium *et al.* did not reproduce these findings in black PKD patients (20). These findings are tempered somewhat by the finding of Kausz *et al.* who reports that women in the United States tend to start dialysis at lower level of residual renal function than men (21). Exploration for the biological

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Abbreviations: ARA, arachidonic acid; ALA, α-linolenic acid; CO, corn oil; FO, flax oil; LNA, linoleic acid; ox-LDL, oxidized low density lipoprotein; PCNA, proliferating cell nuclear antigen; PKD, polycystic kidney disease; SDG, secoisolariciresinol dyglycoside.

basis of gender differences has identified potential estrogen-sensitive pathways in inflammatory, vascular, and mesangial responses to renal injury (22–26).

Current research on gender as a determinant of progression of renal injury is yet to consider the possibility that gender exerts a modulating effect on environmental influences such as diet on the progression of renal disease. Major studies of dietary intervention in renal disease, such as the Modification of Diet in Renal Disease Study (27), already challenged by issues of compliance and difficulty in controlling for specific nutrient effects, have not considered or lacked power to consider potential interactions between gender and diet, or alternatively, like the Nurses' Health Study (28), were single-gender in nature. The Han:SPRD-*cy* rat also shows marked sexual dimorphism in an autosomal dominant form of PKD, with much slower progression of histologic and functional renal injury in female animals (29). Castration of male animals slows the progression of the disease, and treatment of female animals with testosterone accelerates the rate of disease progression, although not to same rate as seen in non-castrated male animals (30). Prior interventional studies in this model, including our own, have focused on the more seriously affected male animals, exploiting the ability to both observe and modify a chronic disease course in a relatively short period of time (12–14). We therefore undertook the studies described in this report to test the hypotheses that benefits that we have previously observed of FO and SDG would be additive, and that the response to these dietary interventions would be influenced by animal gender in the Han:SPRD-*cy* rat.

EXPERIMENTAL PROCEDURES

Animals. All animal procedures and care were examined by the University of Manitoba Committee on Animal Use, and were within the guidelines of the Canadian Council on Animal Care. Surviving offspring of known Han:SPRD-*cy* heterozygotes from our own breeding colony were randomly assigned to groups fed FO or corn oil (CO) with or without 20 mg/kg diet supplementation with SDG *ad libitum* at weaning at 3 wk of age. Animals were killed after 12 wk on the diet, and kidney, liver, and serum were collected for analysis. Fasting 6-h urine was collected for protein excretion using metabolic cages on the day prior to tissue harvest. Diets were based on the AIN 93 formula using casein as the protein source (20% by weight) supplemented with 0.3% methionine, corn starch (52% by weight), and dextrose (13% by weight) as the carbohydrate sources and 7% CO or 7% FO (Omega Nutrition, Vancouver). SDG was a kind gift of Dr. Neil Westcott, Agriculture Canada, Saskatoon SK, Canada. The FA analysis of this diet has been previously reported (14). The animals were fed *ad libitum*, as our prior studies have not found differences in intake related to disease status in animals in the earlier stages of disease progression (12–14,31,32).

Histology and immunohistochemistry. Tissue from the left kidney was processed using our previously described methods (13,31) for histologic and immunohistochemical analysis. These studies included hematoxylin and eosin, aniline blue staining for fibrosis, and macrophages (MAB1435, Chemicon International, Temecula, CA). Oxidized LDL (ox-LDL) staining was used as a marker of oxidant injury (33), using a polyclonal antibody (AB3230, Chemicon, Temecula, CA). Animals were classified as affected by one of two experienced observers (NBC, MRO), blinded to dietary intervention on the basis of the characteristic cystic and inflammatory pathology of this disease.

Image analysis. Image analysis procedures were performed with a system consisting of a Spot Junior CCD camera mounted on an Olympus BX60 microscope. The captured images from random stage movement through the sections were subsequently analyzed using Image Pro version 4.5 Package (Media Cybernetics, Silver Spring, MD). The observer was blinded to dietary treatment, although disease status is obvious upon microscopic examination. Raw analysis data were processed as previously described (13,31) to give objective measures of cystic change, fibrosis, interstitial macrophage infiltration, and extent of ox-LDL staining. Measurements of fibrosis and cellular markers were corrected to solid tissue areas of sections so that the presence of empty cystic areas on the section did not lead to an underestimate of these parameters.

Chemistry. Biochemical measurements were performed by an observer blinded to disease status and dietary intervention (E.N.). Serum and urine creatinine, and serum cholesterol, were determined by spectrophotometric methods using Sigma kits (Sigma Chemical Co., St Louis, MO). Urine protein was measured by the brilliant Coomassie Blue method of Bradford.

Gas chromatography. Lipids were extracted for GC analysis using a modified Folch extraction procedure, as we have previously described (13,31,34). Prior to analysis, samples were redissolved in 1 mL of dry toluene, mixed with 2.0 mL of 0.5 M sodium methoxide and heated to 50°C for 10 min, then mixed with 0.1 mL of glacial acetic acid, 5 mL of distilled H_2O, and 5 mL of hexane. After vortexing, the mixture was centrifuged at 2500*g* for 10 min, and the hexane fraction was removed. Fresh hexane was added to the remaining solution and the previous steps repeated. The hexane fractions were dried under anhydrous sodium sulfate and evaporated under nitrogen, and the lipid esters were redissolved in 1 mL hexane. GC was performed on a Varian Chrompack 3800 instrument, using a Zebron ZB Wax 30-m column (Phenomenex, Torrance, CA). Total n-6:n-3 ratio was calculated from the sum of proportions of linoleic acid (LNA, 18:2 *c*9,*c*12), γ-linolenic acid (GLA), 20:3 n-6 and arachidonic acid (ARA), divided by the sum of α-linolenic acid (ALA), 20:3 n-3, EPA, and DHA. The ratio of ARA:LNA was calculated as a measure of Δ-6 desaturase activity.

PGE_2 measurement. Approximately 250 mg of kidney was snap frozen in liquid nitrogen immediately upon removal from the animal and subsequently lyophilized. The lyophilized tissue was later reconstituted in 10 mL of Hanks balanced salt solution. The homogenate was incubated at 37°C for 1 h, at which point indomethacin was added and the solution vortexed to stop synthesis (35). After centrifugation, the supernatant was stored

at –80°C until duplicate analysis of PGE_2 using an ELISA (R&D Systems, Minneapolis, MN). To minimize the interference of the Hanks balanced salt solution with the alkaline phosphatase enzyme (according to manufacturer's specifications), standards were reconstituted using this solution as opposed to the buffer provided with the kit. This kit does have cross reactivity with PGE_1 (70%) and PGE_3 (16.3%). As prostanoid production of the 2 series has previously been shown to be very predominant in the kidney, even when diets are enriched with other prostanoid series precursors (36), PGE_2 would represent the major molecule detected by a system that recognizes both PGE_1 and PGE_2.

Statistical analysis. Data was analyzed using a general linear model ANOVA using the Minitab 13 software package. Disease effects on physical and biochemical parameters were analyzed in a model using disease *vs.* normal, FO *vs.* CO, and SDG *vs.* no SDG as variables. Analysis included detection of potential interaction between variables. *Post hoc* comparisons were performed using the Tukey-Kramer method within the same software package. The relationship of renal prostaglandin release to disease state, gender, histologic variables, and FA content was explored with logistic regression using the same software package.

RESULTS

A total of 76 male animals, including 28 without PKD and 48 with PKD, and 79 female animals, including 29 without PKD and 50 with PKD ,were included in the study. The distribution between diet and disease groups is shown in Table 1 for males and Table 2 for females. Animal weights were higher in male animals, as expected, and significantly reduced by PKD, although FO conferred significant protection against this weight loss ($P = 0.012$) (Table 1). In female animals, however, disease was not associated with weight reduction, but SDG produced a small but significant reduction in weight in both normal and PKD animals that was not observed in male animals. Kidney weight was significantly increased by PKD ($P < 0.001$) in both genders, and uninfluenced by dietary therapy (Tables 1 and 2). In male animals, there was a small but significant increase in serum creatinine associated with SDG therapy in normal animals ($P = 0.014$), but a small but just significant decrease in serum creatinine in animals with PKD ($P = 0.05$). In female animals, FO was associated with a small statistically, but not clinically, significant decrease in serum creatinine. (Tables 1 and 2). Protein/creatinine ratio was significantly increased by the presence of PKD in males only, although FO was associated with significant reduction of this ratio in PKD animals of both gender ($P < 0.05$).

Male PKD animals demonstrated significant reductions in cystic change ($P = 0.036$) and macrophage infiltration ($P = 0.001$) in response to FO, as we have described previously, and reduction in macrophages in response to SDG ($P < 0.001$), with no effect of SDG detected on fibrosis, epithelial proliferation, or ox-LDL detection (Figs. 1 and 2). In female PKD animals, however, FO was associated with significant reduction in cyst area ($P = 0.001$), fibrosis ($P = 0.031$), ox-LDL ($P = 0.026$), macrophages ($P < 0.05$), and epithelial proliferation ($P = 0.028$), with SDG associated with reduced ox-LDL detection ($P = 0.011$) and reduced macrophage infiltration ($P = 0.017$) (Figs. 1 and 2).

PGE_2 release was increased by the presence of PKD only in male animals ($P = 0.001$), and reduced by FO in all but normal female animals (male normal $P = 0.003$, male PKD $P < 0.001$, female PKD $P < 0.001$) (Fig. 3). Gender was not significantly related to PGE_2 release in the regression analysis. Macrophage count ($r^2 = 0.082$, $P = 0.004$) and renal arachidonic acid (ARA) ($r^2 = 0.145$, $P < 0.001$) were positively related to PGE_2 release, and renal tissue EPA ($r^2 = 0.30$, $P < 0.001$) was negatively related to PGE_2 release.

In male animals, FO was generally associated with decrease in renal n-6 PUFA and increase in renal n-3 PUFA, as expected (Table 3). Significant interaction between the pres-

TABLE 1
Weight, Renal Function and Biochemical Indices of Male Rats Used in the Study

	Corn oil	Flax oil	Corn oil + SDG	Flax oil + SDG	P for oil effect	P for SDG effect	P for disease
Normal (*n*)	9	9	9	7			
PKD (*n*)	11	12	12	13			
Animal weight (g) for normal group	454 (7.8)	445 (7.8)	453 (7.8)	460 (8.9)	NS	NS	NA
Animal weight (g) for PKD group	430 (7.1)	453 (6.8)	431 (6.8)	439 (6.5)	0.012	NS	0.007
Combined kidney weight (g) for normal group	2.7 (0.27)	2.8 (0.27)	3.1 (0.27)	2.7 (0.31)	NS	NS	NA
Combined kidney weight (g) for PKD group	5.8 (0.25)	6.2 (0.24)	5.8 (0.24)	6.5 (0.23)	NS	NS	<0.0001
Creatinine (μmol/L) for normal group	60 (6.7)	62 (6.7)	66 (6.7)	70 (7.6)	NS	0.014	NA
Creatinine (μmol/L) for PKD group	119 (6.0)	112 (5.8)	102 (5.8)	115 (5.6)	NS	0.05	<0.0001
Urine protein/creatinine ratio (mg/mmol) for normal group	94 (9)	79 (8)	83(8)	72 (10)	NS	NS	NA
Urine protein/creatinine ratio (mg/mmol) for PKD group	131 (29)	90 (28)	163 (28)	93 (27)	<.05	NS	0.04

NA, not applicable; NS, not significant.

TABLE 2
Weight, renal function and biochemical indices of Male Rats Used in the Study

	Corn oil	Flax oil	Corn oil + SDG	Flax oil + SDG	P for oil effect	P for SDG effect	P for disease
Normal (*n*)	8	5	10	6			
PKD (*n*)	15	16	12	17			
Animal weight (g) for normal group	292 (4.8)	313 (6.1)	290 (4.3)	293 (5.5)	NS	0.035	NA
Animal weight (g) for PKD group	298 (3.5)	297 (3.4)	290 (3.9)	289 (3.3)	NS	0.045	NS
Combined kidney weight (g) for normal group	2.1 (0.11)	1.9 (0.13)	1.8 (0.09)	1.8 (0.12)	NS	NS	NA
Combined kidney weight (g) for PKD group	2.8 (0.08)	2.8 (0.07)	2.8 (0.09)	2.7 (0.07)	NS	NS	<0.0001
Creatinine (μmol/L) for normal group	67 (3.9)	71 (4.9)	66 (3.2)	68 (4.4)	NS	NS	NA
Creatinine (μmol/L) for PKD group	65 (2.8)	66 (2.7)	72 (3.1)	65 (2.6)	0.05	NS	NS
Urine protein/creatinine ratio (mg/mmol) for normal group	74 (8)	84 (10)	77(7)	81 (9)	NS	NS	NA
Urine protein/creatinine ratio (mg/mmol) for PKD group	107(10)	97 (10)	100 (11)	93 (10)	<.05	NS	0.009

For abbreviations, see Table 1.

ence of PKD and oil was observed, with *post hoc* analysis confirming that the presence of PKD was associated with a reduction in LNA ($P = 0.042$) and increase in ARA ($P = 0.031$ and ARA:LNA ratio, implying increased Δ6-desaturase activity. FO diet was associated with a reduction in ARA:LNA ratio, implying a reduction in Δ6-desaturase activity. SDG had no effect on renal PUFA content in males. Female animals showed a similar profile of FO effects to the males (Table 4), but SDG feeding was associated with reduction in renal content of LNA and ALA, which demonstrated a significant interaction that *post hoc* analysis attributed to an apparent increase in ARA and EPA, respectively, implying increased Δ6-desaturase activity, that was greater in CO-fed animals.

Both male and female rats demonstrated the expected effects of FO on hepatic PUFA content as described in our previous studies (14), so detailed data are not presented. Interestingly, in contrast to renal tissue, SDG was associated with higher hepatic ARA content as seen in male animals only ($P = 0.02$). Neither SDG nor disease state influenced hepatic FA content in female animals, nor was there interaction between dietary components or between diet and disease. The lack of difference in hepatic PUFA content suggests that the observed differences in renal content are not due to unrecognized differences in intake.

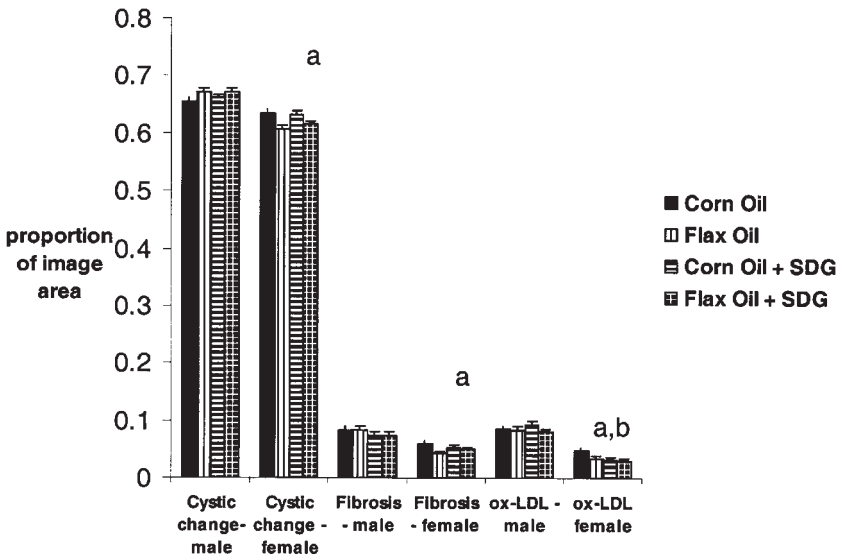

FIG. 1. Morphometric assessment of cystic change, renal fibrosis, and ox-LDL staining of renal tissue of male and female animals with PKD. Results are expressed as an average fraction of section area demonstrating staining or cystic change as applicable. Fibrosis and ox-LDL are corrected to non-cystic area. a, significant effect of dietary oil within the gender group ($P < 0.05$); b, significant effect of SDG within gender group. Bars represent SEM.

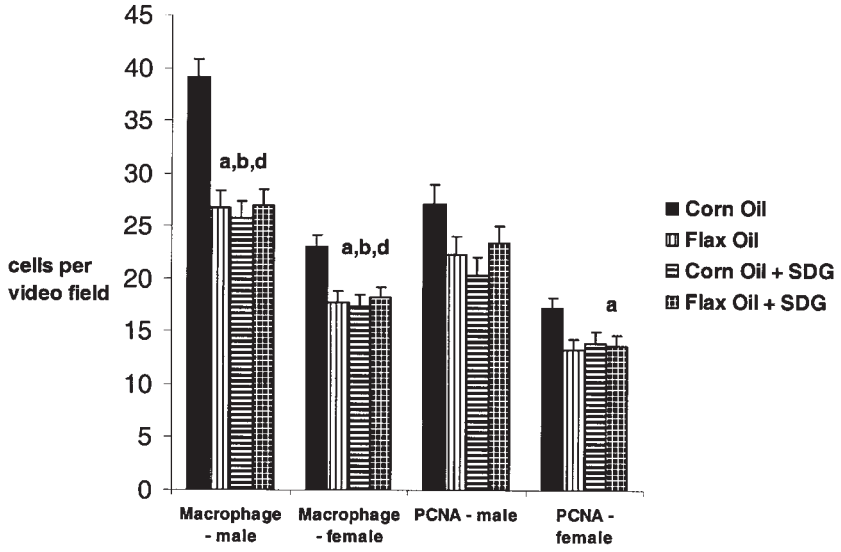

FIG. 2. Morphometric assessment of macrophage infiltration as a marker of inflammation and PCNA as a marker of proliferation. Results are expressed as the average number of positive staining cells per high-power video field, corrected to non-cystic tissue area. a, significant effect of dietary oil within the gender group ($P < 0.05$); b, significant effect of SDG within gender group ($P < 0.05$); d, significant interaction detected with SDG having a greater effect in post hoc testing in the presence of CO. Bars represent SEM.

DISCUSSION

Our results generally confirm the benefit of flax derivatives demonstrated in our earlier studies. The relative differences in beneficial effect between male and female animals could arise from either a direct relationship between gender and the biological consequences of feeding flax derivatives, or from a relationship between the feeding intervention and the stage of disease progression at a specified age, which clearly differs between genders in this model.

In the context of the Han:SPRD-*cy* rat, gender-dependent effects might include deleterious effects of androgens, or benefi-

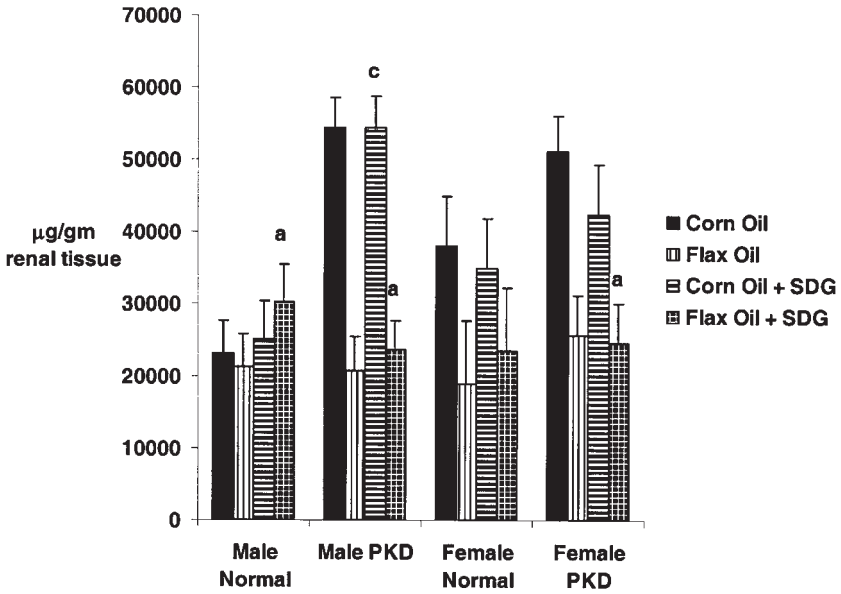

FIG. 3. Renal prostaglandin release expressed as µg/g of tissue. a, significant effect of dietary oil within the gender and disease group ($P < 0.01$); c, significant effect of PKD within the gender group ($P < 0.01$). Bars represent SEM.

TABLE 3
Kidney PUFA Analysis for– Male Rats

	Corn oil	Flax oil	Corn oil + SDG	Flax oil + SDG	P for oil effect	P for SDG effect	P for disease
18:2n-6 for normal group	19.3 (1.2)	14.6 (1.2)	17.5 (1.2)	13.7 (1.4)	0.002	NS	NA
18:2n-6 for PKD group	14.7 (1.4)	13.3 (1.2)	15.7 (1.3)	13.0 (1.2)	NS	NS	0.042
18:3n-3 for normal group	0.2 (1.0)	7.7 (1.0)	0.1 (1.0)	8.4 (1.2)	<0.001	NS	NA
18:3n-3 for PKD group	0.1 (1.4)	7.1 (1.3)	0.2 (1.3)	7.4 (1.3)	<0.001		NS
20:4n-6 for normal group	15.4 (2.5)	12.3 (2.5)	23.4 (2.5)	10.9 (2.8)	0.005	NS	NA
20:4n-6 for PKD group	25.1 (2.2)	13.2 (2.0)	24.3 (2.0)	12.9 (1.9)	<0.001	NS	0.031
20:5n-3 for normal group	0.03 (0.34)	3.33 (0.34)	0.04 (0.34)	3.12 (0.38)	<0.001	NS	NA
20:5n-3 for PKD group	0.05 (0.35)	3.40 (0.32)	0.06 (0.32)	3.35 (0.31)	<0.001	NS	NS
22:5n-3 for normal group	0.063 (0.07)	0.88 (0.07)	0.092 (0.07)	0.88 (0.07)	<0.001	NS	NA
22:5n-3 for PKD group	0.15 (0.22)	2.06 (0.20)	0.15 (0.20)	2.05 (0.19)	<0.001	NS	<0.001[b]
22:6n-3 for normal group	0.46 (0.14)	1.32 (0.14)	0.68 (0.14)	1.22 (0.16)	<0.001	NS	NA
22:6n-3 for PKD group	0.54 (0.14)	1.28 (0.13)	0.54 (0.13)	1.38 (0.13)	<0.001	NS	NS
n6:n3 ratio for normal group	49.0 (2.2)	2.2 (2.2)	43.6 (2.5)	1.9 (2.2)	<0.001	NS	NA
n6:n3 ratio for PKD group	48.0 (2.9)	2.1 (2.6)	45.4 (2.6)	2.0 (2.5)	<0.001	NS	NS
ARA:LNA for normal group	0.9 (0.2)	1.6 (0.2)	0.8 (0.2)	0.8 (0.3)	NS	NS	NA
ARA:LNA for PKD group	2.0 (0.2)	1.0 (0.2)	1.9 (0.2)	1.0 (0.2)	<0.001	NS	NS

Results other than ratios are percent total lipid. b, statistically significant interaction between disease and oil detected at $P < 0.01$, refer to text for comment For abbreviations, see Table 1.

cial effects of estrogens, either naturally occurring or in the form of derivatives of SDG. Nagao *et al.* have recently shown that male Han:SPRD-*cy* rats demonstrate significantly increased renal androgen receptor (AR) expression, which is reduced by castration and restored by exogenous androgen administration (37). They also examined the phospho-extracellular signal–regulated kinase 1/2 (P-ERK) pathways that have been linked to up-regulated cell proliferation in the Han:SPRD-*cy* rat model and found evidence of a relationship between AR expression and P-ERK that suggested that P-ERK was abnormally regulated with or without androgens but androgens had an additive role. Interestingly, although our earlier studies in younger male animals did demonstrate an effect of both FO and SDG on renal epithelial cell proliferation as detected by PCNA, the older male animals in this study demonstrated no dietary effect of either FO or SDG on proliferation, whereas FO reduced proliferation in females to an extent comparable to that seen in younger males (14). As the proliferative pathway seems to be predominant mediator of androgen effect, it seems unlikely that the gender differences observed here arise from androgen antagonism. This would also be in accord with the findings of Cowley *et al.* who confirmed the earlier findings of Zeier *et al.* that castration slowed histologic disease progression in males, and that the rate of progression was restored by testosterone administration (30,38). Testosterone administration to ovarectomized or sham operated female animals, however, in the later study accelerated the rate of disease progression, but not to the degree normally seen in males. The inference is that gender influences disease progression through both sex steroid–dependent and nondependent effects. Our results suggested that baseline renal PGE_2 production was

TABLE 4
Kidney PUFA Analysis for Female Rats

	Corn oil	Flax oil	Corn oil + SDG	Flax oil + SDG	P for oil effect	P for SDG effect	P for disease
18:2n-6 for normal group	18.1 (1.5)	11.5 (1.9)	21.1 (1.4)	13.9 (1.7)	<0.001	NS	NA
18:2n-6 for PKD group	20.1 (1.0)	12.7 (0.9)	14.9 (1.1)	12.8 (0.9)	<0.001	0.004[a]	NS
18:3n-3 for normal group	0.2 (0.7)	8.5 (0.6)	0.2 (0.9)	3.8 (0.8)	<0.001	0.006[a]	NA
18:3n-3 for PKD group	0.2 (0.7)	5.0 (0.7)	0.1 (0.8)	6.8 (0.6)	<0.001	NS	NS
20:4n-6 for normal group	19.7 (2.7)	7.9 (2.4)	15.0 (3.5)	14.8 (3.2)	NS	NS	NA
20:4n-6 for PKD group	16.3 (1.8)	13.9 (1.7)	25.5 (2.1)	12.1 (1.7)	<0.001	NS a	NS
20:5n-3 for normal group	0.10 (0.35)	3.03 (0.44)	0.02 (0.32)	5.97 (0.41)	<0.001	0.001[a]	NA
20:5n-3 for PKD group	0.05 (0.32)	4.84 (0.37)	0.03 (0.31)	4.23 (0.30)	<0.001	NS	NS
22:5n-3 for normal group	0.10 (0.08)	0.70 (0.10)	0.07 (0.07)	1.27 (0.09)	<0.001a	0.003[a]	NA
22:5n-3 for PKD group	0.07 (0.09)	1.44 (0.09)	0.10 (0.11)	1.21 (0.09)	<0.001	NS	0.026
22:6n-3 for normal group	1.01 (0.26)	1.53 (0.23)	0.91 (0.33)	2.99 (0.30)	<0.001	0.024[a]	NA
22:6n-3 for PKD group	0.73 (0.15)	2.29 (0.15)	1.09 (0.18)	1.97 (0.15)	<0.001	NS	NS
n6:n3 ratio for normal group	31.0 (3.5)	1.5 (4.5)	37.6 (3.2)	2.0 (4.1)	<0.001	NS	NA
n6:n3 ratio for PKD group	39.3 (1.8)	2.0 (1.8)	31.9 (2.1)	1.8 (1.7)	<0.001	0.045	NS
ARA:LNA for normal group	1.3 (0.3)	0.7 (0.3)	0.9 (0.2)	1.1 (0.3)	NS	NS	NA
ARA:LNA for PKD group	1.0 (0.2)	1.1 (0.2)	1.9 (0.2)	0.9 (0.1)	0.005	0.021[a]	NS

Results other than ratios are percent total lipid. a, statistically significant interaction between oil and SDG diets detected at $P < 0.01$, refer to text for comment. For abbreviations, see Table 1.

higher in female animals, an effect previously observed in SHR rats by Sullivan *et al.* (39). In those studies, the authors reported increased PGE metabolite excretion and renal expression of PGE synthase in females, and increases in response to orchidectomy in male animals. The levels of metabolite excretion and PGE synthase activity observed in males after orchidectomy, however, remained lower than both ovarectomized and intact females. Yang *et al.* have reported that female gender is protective against hypertension and renal injury in cyclooxygenase 2–deficient mice, although this effect was only specific to one genetic background (40). PGE_2 is know to act through four G-protein coupled receptors, and evidence is accumulating that the different and sometimes contradictory effects of PGE_2 in the kidney, such as vasodilatation and salt excretion *vs.* vasoconstriction and activation of the renin-angiotensin system, may be mediated interactions with different receptors (41). The EP4 receptor in particular has been linked to interactions that are relevant to the pathogenesis of renal injury in this model. Vukicevic *et al.* reported reduced glomerular and tubulo-interstitial injury in nephrotoxic renal injury in rats treated with a selective EP4 receptor agonist (42). The distribution and expression of these receptors in the kidney are incompletely characterized, and the possibility of gender influence on that effect remains to be explored

There are a number of estrogen-mediated pathways that may have a moderating influence on renal injury, which may be relevant to SDG effects. Estrogen downregulates the key inflammatory cytokine, monocyte chemoattractant protein 1 (MCP-1) (43,44). In a prior study, we have demonstrated upregulation of MCP-1 in the Han:SPRD-*cy* rat (45), with a reduction in MCP-1 RNA expression in association with severe dietary protein restriction. Krepinsky *et al.* have shown that estrogen inhibits strain-activated MAP-kinase expression in mesangial cells, and posit that this may reduce maladaptive glomerular responses to renal injury (46). Estrogen-progesterone combination therapy reduces expression of endothelin-1 and renal injury in the DOCA-salt hypertension rat model (24). An estrogen receptor–mediated role in cystic disease has been the subject of speculation in the pathogenesis of acquired cystic disease of the kidney, an entity seen in the failed kidneys of patients who are supported for many years on dialysis, which also demonstrates male preponderance. Concolino *et al.* have suggested that the decreased androgen/estrogen ratio of uremia results in greater epithelial stimulation in males as the male tissues are less adapted to the high-estrogen environment, but this is yet to be supported by experimental data in human or animal models (47). Other beneficial effects ascribed to estrogen include antioxidant effects, favorable shift in the collagen synthesis/degradation balance, and suppression of pro-inflammatory or proliferative cytokines (48). In this study, it is interesting to note that SDG had more histologic effect in the female animals, where both a change in inflammation and tissue ox-LDL content was detected whereas only the anti-inflammatory effect was seen in the males. It is possible that the direct antioxidant effect of SDG alone was not sufficient to produce a detectable effect in the males, whereas the combination of the direct effect of SDG and the indirect effect of estrogenic metabolites in the females resulted in a detectable difference. It is also of interest the SDG treatment significantly interacted with FO diet with respect to renal PUFA composition in females, but not in males. This effect was specific to renal tissue, whereas the composition of hepatic tissue was consistent with expectations created by the nature of the dietary lipid source. The implication is that SDG has effects on renal lipid composition that are gender-specific and may relate to the level of expression of renal estrogen receptors and the availability of estrogenic ligands.

Another explanation of our findings is that the maximal benefit of flax-based interventions occurs only in the early course of histologic renal injury. Our prior studies of flaxseed, FO, and SDG all involved feeding male animals from weaning until 9 or 11 wk of age (13,31,32), whereas this study fed until 15 wk, when disease is more advanced, which is reflected in the higher cyst scores in this study. By analogy based on morphometric scoring of cystic change in our different studies, which we have done as early as 2 and 4 wk of age, the disease in the female animals is probably at a level comparable to male animals less than half their age. The range and magnitude of effect we saw in the female animals is certainly comparable to our results in younger males, whereas our results in the older males in this study were considerably less impressive.

Gender did not influence the impact of FO on renal prostanoids release, an outcome that also was not influenced by SDG. The other outcome that was clearly unrelated to gender was the reduction of proteinuria, considered a marker of glomerular injury, by FO. This benefit of n-3 PUFA enrichment has been described in both rodent and human studies, and may be a direct effect of the modification of prostanoid synthesis (15,16,49,50). The significance of this and other effects of histologic improvements in renal injury needs to be studied in longer-term, and therefore unfortunately more expensive, survival studies.

Our study has a number of implications for research and application of dietary modification of renal disease. Our findings continue to add weight to the concept that whole-of-life dietary pattern may be more important in the relationship of diet to renal injury than dietary changes late in the course of disease, thus supporting the general findings of most human studies. More importantly, our studies reinforce that there may be a biologic basis to consider gender as not only a variable in the natural history of illness, but also in the biology of therapeutic strategies.

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